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(54) Title: COMPOSITION AND METHOD FOR <i>IN VIVO</i> AND <i>IN VITRO</i> ATTENUATION OF GENE EXPRESSION USING DOUBLE STRANDED RNA			
(57) Abstract			
Introduction of double stranded RNA into cells, cell culture, organs and tissues, and whole organisms, particularly vertebrates, specifically attenuates gene expression.			
<div style="display: flex; flex-direction: column; align-items: flex-start;"> <div style="margin-bottom: 20px;"> <p><b>A</b></p> <p>pEGFP-N1</p> <p>dsRNA</p> </div> <div style="margin-bottom: 20px;"> <p><b>B</b></p> <p>Zf-T</p> <p>dsRNA</p> </div> <div> <p><b>C</b></p> <p>Pax6.1</p> <p>dsRNA</p> </div> </div>			

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**COMPOSITION AND METHOD FOR *IN VIVO* AND *IN VITRO***  
**ATTENUATION OF GENE EXPRESSION**  
**USING DOUBLE STRANDED RNA**

This application claims the benefit of U.S. Provisional Application Serial No. 60/117,635, filed January 28, 1999.

**Statement of Government Rights**

This invention was made with government support under grants from the United States Public Health Service, Grant Nos. HL36059, HL51533, and HD17063. The U.S. Government has certain rights in this invention.

**Background of the Invention**

Double-stranded RNA has been shown to attenuate specific gene expression in *C. elegans*, *Drosophila* and *Trypanosoma brucei* (M. Montgomery, et al., *Proc. Natl. Acad. Sci. U.S.A.* 95, 15502-15507 (1998); J. Kennerdell et al., *Cell* 95, 1017-1026 (1998); H. Ngo et al., *Proc. Natl. Acad. Sci. U.S.A.* 95, 14687-14692 (1998)). The types of genes attenuated in these invertebrates include some encoding transcription factors and others that encode growth factor receptors. There is also evidence that double-stranded RNA may effectively silence gene expression in plants (M. Wassenegger et al., *Plant. Mol. Biol.* 37, 349-362 (1998); P. Watergiyse et al., *Proc. Natl. Acad. Sci. U.S.A.* 95, 13959-13964 (1998)). Application of this method to vertebrates would be an extremely useful tool for the study of vertebrate developmental genetics and has numerous medical implications as well, however researchers have heretofore been unable to obtain successful gene silencing in vertebrates using this method.

A definitive mechanism through which double-stranded RNA effects gene silencing remains has not been identified (M. Montgomery et al., *Trends Genet.* 14, 255-258 (1998)). Recently, Montgomery et al. reported that double-stranded RNA induces specific RNA degradation in nematodes (*Proc. Natl. Acad. Sci. U.S.A.* 95, 15502-15507 (1998)). This conclusion was based upon the

fact that DNA sequences in the targeted regions of the gene were not altered and that 100% of the F2 generation reverted to the wild type phenotype. In addition, *C. elegans* has a unique genetic organization. Genes in this animal are organized in operons in which a single promoter controls expression of a number of genes.

5 They showed that the double-stranded RNA affects only expression of the targeted gene. In contrast, however, others have observed heritable effects of double-stranded RNA on the expression of a number of genes in *C. elegans*, suggesting that more than one mechanism may be involved in double-stranded RNA-mediated inhibition of gene activity (H. Tahara, *Science* 28, 431-432  
10 (1998)).

In transgenic plants, co-suppression of gene expression can be mediated through rapid degradation of the mRNA produced by the targeted gene (D. Smyth, *Curr. Biol.* 7, R793-795 (1997)). Others have shown that double-stranded RNA-dependent sequence-specific methylation may mediate the long-  
15 term effects of co-suppression in plants. Such a methylase may also be dependent on transcription of the targeted sequence since double-stranded RNA targeted to promoter regions in nematode had no apparent effect on transcription.

### **Summary of the Invention**

20 The present invention provides a method for attenuating gene expression in a cell using gene-targeted double-stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene. The cell into which the dsRNA is introduced can be derived from or contained in any organism (e.g., plant,  
25 animal, protozoan, virus, bacterium, or fungus). Gene expression can be attenuated in a whole organism, an organ or tissue of an organism, including a tissue explant, or in cell culture. Preferably, the cell is a vertebrate cell, but the invention is not limited to vertebrates. Double-stranded RNA is introduced directly into the cell or, alternatively, into the extracellular environment from  
30 which it is taken up by the cell. Inhibition is specific for the targeted gene. The targeted gene can be a chromosomal gene or an extrachromosomal gene. For

example, the targeted gene may be present in the genome of the cell into which the dsRNA is introduced, or in the genome of a pathogen, such as a virus, a bacterium, a fungus or a protozoan, which is capable of infecting such cell. The targeted gene can be an endogenous gene or a foreign gene. Depending on the particular target gene and the dose of dsRNA delivered, the method may partially or completely inhibit expression of the gene in the cell. The expression of two or more genes can be attenuated concurrently by introducing two or more double stranded RNAs into the cell in amounts sufficient to attenuate expression of their respective target genes. Double stranded RNAs that are administered "concurrently" are administered, together or separately, so as to be effective at generally the same time.

In another aspect, the invention provides a method for attenuating the expression of a target gene in an organism that involves introducing a double stranded RNA into an embryo in an amount sufficient to attenuate expression of the target gene, then growing the embryo into a fully developed organism, e.g., an adult organism, in which expression of the target gene is attenuated. Optionally, a phenotypic change in the organism associated with attenuated expression of the target gene can be identified.

In another aspect, the invention provides a method for attenuating the expression of a target gene in a tissue explant that involves explanting a tissue from an organism then introducing a double stranded RNA into a cell of the tissue explant in an amount sufficient to attenuate expression of the target gene. Optionally, the tissue explant exhibiting attenuated expression of the target gene is implanted back into the organism or is implanted into a different organism. Also optionally, a phenotypic change in the tissue explant associated with attenuated expression of the target gene can be identified.

In yet another aspect, the invention provides a method for attenuating the expression of a target gene in a cell that includes annealing two complementary single stranded RNAs in the presence of potassium chloride to yield double stranded RNA; contacting the double stranded RNA with RNase to purify the double stranded RNA by removing single stranded RNA; and introducing the

purified double stranded RNA into the cell in an amount sufficient to attenuate expression of the target gene.

The invention further provides a method for treating or preventing a disease or infection in a mammal. Double stranded RNA is administered to the mammal in an amount sufficient to attenuate expression of a target gene, the expression of which is associated with the disease or infection. The method can be used to treat or prevent a viral infection, in which case the double stranded RNA is an antiviral double stranded RNA that attenuates the expression of a viral gene. Alternatively, the method can be used to treat or prevent cancer, in which case the double stranded RNA is an antitumor double stranded RNA, or to treat an autosomal dominant genetic disease such as Huntington's chorea, in which case the double stranded RNA attenuates the expression of an allele of a gene that is associated with the disease. Concurrent inhibition of multiple genes is advantageous to treat diseases associated with multiple genes, or to treat two or more diseases or infections concurrently.

The method of the invention can further be used to reduce or prevent the rejection response to transplant tissue. A double stranded RNA that attenuates the expression of a gene in the transplant tissue that can elicit an immune response in the recipient is administered to the transplant tissue. Preferably, the transplant tissue is hepatocytes.

Also provided by the invention is a vertebrate cell that contains a double stranded RNA having a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of a target gene. The vertebrate cell is preferably a fish cell, a murine cell, a bird cell or a human cell. A vertebrate that contains the vertebrate cell of the invention is also provided.

The invention also provides a kit that includes reagents for attenuating the expression of a target gene in a cell. The kit contains a DNA template that has two different promoters (preferably a T7 promoter, a T3 promoter or an SP6 promoter), each operably linked to a nucleotide sequence. Two complementary single stranded RNAs can be transcribed from the DNA template, which can be annealed to form a double stranded RNA effective to attenuate expression of the

target gene. The kit optionally contains amplification primers for amplifying the DNA sequence from the DNA template and nucleotide triphosphates (i.e., ATP, GTP, CTP and UTP) for forming RNA. Also optionally, the kit contains two RNA polymerases, each capable of binding to a promoter on the DNA template and causing transcription of the nucleotide sequence to which the promoter is operably linked; a purification column for purifying single stranded RNA, such as a size exclusion column; one or more buffers, for example a buffer for annealing single stranded RNAs to yield double stranded RNA; and RNase A or RNase T for purifying double stranded RNA.

#### **Brief Description of the Drawings**

Figure 1 is a schematic of double-stranded RNA targeted to (a) green fluorescent protein(GFP); (b) Zf-T; or (c) Pax6.1; the location of the sequences that were used as templates to produce the GFP, Zf-T, or Pax6.1 double-stranded RNA are indicated as dsRNA and the starting and ending bases are indicated for each.

Figure 2 is an agarose gel that demonstrates annealing of double-stranded Zf-T RNA. Sense and antisense Zf-T RNA strands (lane 1) were treated with RNase A before (lane 2) and after (lane 4) annealing. Each lane was loaded with 0.5 µg of treated (lanes 2 and 4) or untreated RNA (lane 1 and 3).

Figure 3 shows the effect of GFP double-stranded RNA injection on the transient expression of GFP in zebrafish embryos. Columns A and C show the same field of embryos by light microscopy as seen under fluorescence in columns B and D, respectively. Columns A and B show embryos injected at the single cell stage while columns C and D show embryos injected at the 16-cell stage. Rows 1-3 are designated by treatment to the left as injected with the GFP expression vector alone (row 1), GFP expression vector with control double-stranded RNA (row 2), or GFP expression vector with GFP double-stranded RNA (row 3). The embryo shown in row 4 was injected at the single cell stage with GFP and Zf-T double-stranded RNA. While the zebrafish yolk does show

some autofluorescence at higher magnifications, it is not apparent at the magnification shown in columns A and B. The yolk fluorescence seen here is from the GFP expression vector and is specifically attenuated by double-stranded RNA to GFP. On the other hand double-stranded RNA targeted to Zf-T does not interfere with GFP expression and since this embryo shows the *ntl* phenotype, the presence of the GFP expression vector does not attenuate the function of the Zf-T double-stranded RNA.

Figure 4 shows the *ntl* phenocopy produced by microinjection of double-stranded Zf-T RNA into single cell embryos. As shown in Column A, no abnormal phenotypes were apparent subsequent to injection of Zf-T sense or antisense RNA. The injection of double-stranded control RNA also produced no apparent phenotypes. Embryos injected with double-stranded Zf-T RNA exhibited greatly reduced tails and their somites lacked the typical chevron shape of those in the wild type embryos. The embryos shown are 5 days old. As shown in Column B, *in situ* hybridizations demonstrate that 9 hour embryos lack Zf-T expression following double-stranded Zf-T RNA injection into single cell embryos, although sense RNA, antisense RNA, and double-stranded control RNA injection had little effect on Zf-T expression. Scale bars=100µm.

Figure 5 shows the effect of Zf-T double-stranded RNA injection on development of the notochord in 24 hour zebrafish embryos. Wildtype zebrafish and embryos injected with the control double-stranded RNA developed an obvious notochord. *Ntl* mutant embryos lacked a notochord along the entire body axis. Twenty-one per cent of the embryos injected with the Zf-T double-stranded RNA also lacked a notochord along the entire body axis. Another 60% lacked notochord in the posterior portion of the body axis. Scale bars=100µm.

Figure 6 shows the effect of Zf-T double-stranded RNA injection on development of the somites in zebrafish embryos. Both (a) wild type zebrafish embryos and (b) those injected with control double-stranded RNA developed characteristic chevron-shaped somites, whereas (c) zebrafish embryos that had been injected with double-stranded Zf-T RNA developed somites that lacked the typical chevron shape. Scale bars=100µm.



Figure 7 shows the effect of Zf-T double-stranded RNA injection on the expression of *sonic hedgehog*. RNA *in situ* hybridizations for *shh* show a one-cell-wide row of cells in the floorplate that are labeled along the length of the trunk and tail in wild type embryos and embryos injected with a control double-stranded RNA; (a) is a top view; (b) is a side view; and (c) is a cross-section. In embryos injected with Zf-T double-stranded RNA, the floorplate has expanded and expression of *shh* is 3-4 cells wide, similar to the expression found in the *ntl* mutant. Scale bars=100µm.

Figure 8 shows the effect of Zf-T double-stranded RNA injection on the expression of *floating head*: (a) side views and (b) dorsal views of tailbud stage zebrafish embryos. RNA *in situ* hybridizations show that *flh* is expressed in an axial stripe in the wild type embryos and in those injected with the control double-stranded RNA. An embryo injected with Zf-T double-stranded RNA shows diffuse *flh* expression in the tailbud and intense expression in the anterior nervous system, while expression in the body axis was greatly diminished. Scale bars=100µm.

Figure 9 shows (a) the range of phenotypes observed in 72 hour zebrafish embryos following injection of Zf-T double-stranded RNA; (b) 3 embryos with a complete phenotype; histological sections were used to confirm that the notochord was absent; and (c) one embryo with a partial phenotype which was confirmed by histological section to have an anterior notochord.

Figure 10 shows that the absence of Pax6.1 expression results in severe abnormalities of head development; (a) 48 hour embryos injected with double-stranded control RNA had normal phenotypes. Microinjection of double-stranded Zf-Pax6.1 RNA into single cell zebrafish embryos resulted in 25% of the embryos having reduced eyes often accompanied by diminished and disorganized forebrains; (b) *in situ* hybridizations demonstrated that 24 hour embryos lack Zf-Pax6.1 expression following double-stranded Zf-Pax6.1 RNA injection into single cell embryos, although double-stranded control RNA injection had no apparent effect on Zf-pax6.1 expression. Scale bars=100 µm.

Figure 11 shows that co-injection of the Zf-T and Zf-Pax6.1 double-stranded RNA. generates embryos with defective notochords and eyes; (a) at 5 days old, zebrafish embryos show significant defects in the eyes and tail; (b) cross sections of 48 hour embryos show that these defects included diminished eye and brain structures and (c) diminished or lacking notochord along with  
5 diminished or lacking notochord along with disorganized somites in the tail. Scale bars=100  $\mu$ m.

Figure 12 shows the effect of HirA double-stranded RNA on the expression of HirA in explanted chick cardiac neural crest tissue.

Figure 13 shows the effect of GFP double-stranded RNA injection on  
10 transient expression of GFP in murine cell culture.

### **Detailed Description**

The present invention provides a method for gene silencing in organisms and cells, especially vertebrates, using gene-specific double-stranded RNA. The  
15 ability to use double-stranded RNA to specifically block expression of particular genes in a multicellular setting both *in vivo* and *in vitro* has broad implications for the study of developmental genetics. Equally important, it opens up a host of new medical applications. Examples include the construction of anti-viral agents, anti-tumor agents, and therapeutics designed to block expression of  
20 specific alleles of genes that have been implicated in autosomal dominant genetic diseases such as Huntington's chorea. As another example, transplant rejection could be managed prior to transplantation by *in vitro* incubation of the tissues to be transplanted, such as hepatocytes, with an agent designed to block expression of genes associated with the generation of the host immune response.

25 The method of the present invention allows for attenuation of gene expression in a cell. "Attenuation of gene expression" can take the form of partial or complete inhibition of gene function. Mechanistically, gene function can be partially or completely inhibited by blocking transcription from the gene to mRNA, or by blocking translation of the mRNA to yield the protein encoded  
30 by the gene, although it should be understood that the invention is not limited to any particular mechanism of attenuation of gene expression. Inhibition of gene

function is evidenced by a reduction or elimination, in the cell, of the activity associated with the protein encoded by the gene. Whether and to what extent gene function is inhibited can be determined using methods known in the art. For example, in many cases inhibition of gene function leads to a change in

5 phenotype which is revealed by examination of the outward properties of the cell or organism or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA),

10 other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta

15 glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin,

20 methotrexate, phosphinothricin, puromycin, and tetracyclin.

Attenuation of gene expression can be quantified, and the amount of attenuation of gene expression in a treated cell compared to a cell not treated according to the present invention can be determined. Lower doses dsRNA may result in inhibition in a smaller fraction of cells, or in partial inhibition in cells.

25 In addition, attenuation of gene expression can be time-dependent; the longer the period of time since the administration of the dsRNA, the less gene expression may be attenuated. Attenuation of gene expression can occur at the level of transcription (i.e., accumulation of mRNA of the targeted gene), or translation (i.e., production of the protein encoded by the targeted gene). For example,

30 mRNA from the targeted gene can be detected using a hybridization probe having a nucleotide sequence outside the region selected for the inhibitory

double-stranded RNA, and translated polypeptide encoded by the target gene can be detected via Western blotting using an antibody raised against the polypeptide. It should be noted that the method of the invention is not limited to any particular mechanism for reducing or eliminating cellular protein activity; indeed, as noted above, it is not yet fully understood how the introduction of dsRNA into a cell causes attenuation of expression of the targeted gene, nor is it known whether single or multiple mechanisms are at work.

The attenuation of gene expression achieved by the method of the invention is specific for the targeted gene. In other words, the dsRNA inhibits the target gene without manifest effects on other genes of the cell. Additionally, the inhibition of the function of specific genes preferably, although not necessarily, passes through the germline.

#### *Targeted gene*

Any gene being expressed in a cell can be targeted. A gene that is expressed in the cell is one that is transcribed to yield an mRNA and, optionally, a protein. The targeted gene can be chromosomal (i.e., genomic) or extrachromosomal. It may be endogenous to the cell, or it may be a foreign gene (i.e., a transgene). The foreign gene can be integrated into the host genome, or it may be present on an extrachromosomal genetic construct such as a plasmid or a cosmid. The targeted gene can also be derived from a pathogen, such as a virus, bacterium, fungus or protozoan, which is capable of infecting an organism or cell.

The cell containing the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies. Examples of vertebrates include fish and

mammals, including cattle, goat, pig, sheep, hamster, mouse, rat, and human; invertebrate animals include nematodes and other worms, *Drosophila*, and other insects. Preferably, the cell is a vertebrate cell.

The cell having the target gene may be from the germ line or somatic,  
5 totipotent or  
pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell can be a gamete or an embryo; if an embryo, it can be a single cell embryo or a constituent cell or cells from a multicellular embryo. The term "embryo" thus also includes fetal tissue. The cell having the  
10 target gene may be an undifferentiated cell, such as a stem cell, or a differentiated cell, such as from a cell of an organ or tissue, including fetal tissue, or any other cell present in an organism. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages,  
15 neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

#### *Double-stranded RNA*

20 The dsRNA is formed from one or more strands of polymerized ribonucleotide. When formed from only one strand, it takes the form of a self-complementary hairpin-type molecule that doubles back on itself to form a duplex. When formed from two strands, the two strands are complementary RNA strands. The dsRNA can include modifications to either the phosphate-  
25 sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Likewise, bases may be modified to block the activity of adenosine deaminase.

The nucleotide sequence of the dsRNA is defined by the nucleotide  
30 sequence of its targeted gene. The dsRNA contains a nucleotide sequence that is essentially identical to at least a portion of the target gene; preferably the dsRNA

contains a nucleotide sequence that is completely identical to at least a portion of the target gene. It should be understood that in comparing an RNA sequence to a DNA sequence, an "identical" RNA sequence will contain ribonucleotides where the DNA sequence contains deoxyribonucleotides, and further that the RNA  
5 sequence will contain a uracil at positions where the DNA sequence contains thymidine. More preferably, the dsRNA that is completely identical to at least a portion of the target gene does not contain any additional nucleotides. The portion of the target gene to which the dsRNA sequence is essentially or completely identical is preferably a sequence that is unique to the genome of the  
10 cell into which the dsRNA is to be introduced.

A dsRNA that is "essentially identical" to at least a portion of the target gene is a dsRNA wherein one of the two complementary strands (or, in the case of a self-complementary RNA, one of the two self-complementary portions) is either identical to the sequence of that portion of the target gene or contains one  
15 or more insertions, deletions or single point mutations relative to the nucleotide sequence of that portion of the target gene. The invention thus has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

Alternatively, a dsRNA that is "essentially identical" to at least a portion of the target gene can be functionally as a dsRNA wherein one of the two  
20 complementary strands (or, in the case of a self-complementary RNA, one of the two self-complementary portions) is capable of hybridizing with a portion of the target gene transcript (e.g., under conditions including 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours;  
25 followed by washing).

The dsRNA nucleotide sequence that is essentially or completely identical to at least a portion of the target gene has a length of preferably at least about 25 bases, more preferably at least about 50 bases, and most preferably at least about 100 bases. The dsRNA nucleotide sequence has a length of  
30 preferably less than about 400 bases, more preferably less than about 300 base, and most preferably less than about 200 bases. It will be understood that the

length of the dsRNA, the degree of homology necessary to affect gene expression, and the most effective dosages can be optimized for each particular application using routine methods.

5     *Synthesis of dsRNA*

Single strands of RNA are synthesized *in vitro*. Preferably, single stranded RNA is enzymatically synthesized from the PCR products of a DNA template, preferably a cloned cDNA template. Provided the sequence of the target gene is known, a cloned cDNA template can be readily made from target  
10 cell RNA using reverse-transcriptase polymerase chain reaction (RT-PCR) to generate a cDNA fragment, following by cloning the cDNA fragment into a suitable vector. Preferably, the vector is designed to allow the generation of complementary forward and reverse PCR products. The vector pGEM-T (Promega, Madison WI) is well-suited for use in the method because it contains  
15 a cloning site positioned between oppositely oriented promoters (i.e., T7 and SP6 promoters; T3 promoter could also be used). After purification of the PCR products, complementary single stranded RNAs are synthesized, in separate reactions, from the DNA templates via RT-PCR using two different RNA polymerases (e.g., in the case of pGEM-T, T7 polymerase and SP6 polymerase).  
20 RNase-free DNase is added to remove the DNA template, then the single-stranded RNA is purified. Single strands of RNA can also be produced enzymatically or by partial/total organic synthesis. The use of *in vitro* enzymatic or organic synthesis allows the introduction of any desired modified ribonucleotide. The RNA strands may or may not be polyadenylated; and the  
25 RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. Preferably, purification of RNA is performed without the use of phenol or chloroform.

Double stranded RNA is formed *in vitro* by mixing complementary single stranded RNAs, preferably in a molar ratio of at least about 3:7, more  
30 preferably in a molar ratio of about 4:6, and most preferably in essentially equal molar amounts (i.e., a molar ratio of about 5:5). Preferably, the single stranded

5 RNAs are denatured prior to annealing, and the buffer in which the annealing reaction takes place contains a salt, preferably potassium chloride. Prior to administration, the mixture containing the annealed (i.e., double stranded) RNA is preferably treated with an enzyme that is specific for single stranded RNA (for example, RNase A or RNase T) to confirm annealing and to degrade any remaining single stranded RNAs. Addition of the RNase also serves to excise any overhanging ends on the dsRNA duplexes.

*Delivery of dsRNA to a cell*

10 Double stranded RNA can be introduced into the cell in a number of different ways. For example, in the case of an embryo, the dsRNA is conveniently administered by microinjection; other methods of introducing nucleic acids into a cell include bombardment by particles covered by the dsRNA, soaking the cell or organism in a solution of the dsRNA, electroporation  
15 of cell membranes in the presence of the dsRNA, liposome-mediated delivery of dsRNA and transfection mediated by chemicals such as calcium phosphate, viral infection, transformation, and the like. The dsRNA may be introduced along with components that enhance RNA uptake by the cell, stabilize the annealed strands, or otherwise increase inhibition of the target gene. In the case of a cell  
20 culture or tissue explant, the cells are conveniently incubated in a solution containing the dsRNA or lipid-mediated transfection; in the case of a whole animal or plant, the dsRNA is conveniently introduced by injection or perfusion into a cavity or interstitial space of an organism, or systemically via oral, topical, parenteral (including subcutaneous, intramuscular and intravenous  
25 administration), vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration. In addition, the dsRNA can be administered via and implantable extended release device. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the



organism to be affected. The dsRNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant.

Alternatively, dsRNA can be supplied to a cell indirectly by introducing  
5 one or more vectors that encode both single strands of a dsRNA (or, in the case of a self-complementary RNA, the single self-complementary strand) into the cell. Preferably, the vector contains 5' and 3' regulatory elements that facilitate transcription of the coding sequence. Single stranded RNA is transcribed inside the cell, and, presumably, double stranded RNA forms and attenuates expression  
10 of the target gene. Methods for supplying a cell with dsRNA by introducing a vector from which it can be transcribed are set forth in WO 99/32619 (Fire et al., published 1 July 1999). A transgenic animal that expresses RNA from such a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the  
15 appropriate organism. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct.

The dsRNA is typically administered in an amount that allows delivery of at least one copy per cell. The amount of dsRNA administered to a cell, tissue,  
20 or organism depends on the nature of the cell, tissue, or organism, the nature of the target gene, and the nature of the dsRNA, and can readily be optimized to obtain the desired level of gene inhibition. To attenuate gene expression in a single cell embryo, for example, at least about  $0.8 \times 10^6$  molecules of dsRNA are injected; more preferably, at least about  $20 \times 10^6$  molecules of dsRNA are  
25 injected; most preferably, at least about  $50 \times 10^6$  molecules of dsRNA are injected. The amount of dsRNA injected into a single cell embryo is, however, preferably at most about  $1000 \times 10^6$  molecules; more preferably, it is at most about  $500 \times 10^6$  molecules, most preferably, at most about  $100 \times 10^6$  molecules. In the case of administration of dsRNA to a cell culture or to cells in tissue, by  
30 methods other than injection, for example by soaking, electroporation, or lipid-mediated transfection, the cells are preferably exposed to similar levels of

dsRNA in the medium. For example, 8-10  $\mu\text{L}$  of cell culture or tissue can be contacted with about  $20 \times 10^6$  to about  $2000 \times 10^6$  molecules of dsRNA, more preferably about  $100 \times 10^6$  to about  $500 \times 10^6$  molecules of dsRNA, for effective attenuation of gene expression.

5           Once the minimum effective length of the dsRNA has been determined, it is routine to determine the effects of dsRNA agents that are produced using synthesized oligoribonucleotides. The administration of the dsRNA can be by microinjection or by other means used to deliver nucleic acids to cells and tissues, including culturing the tissue in medium containing the dsRNA.

10           *Scientific, industrial and medical applications of the technology*

The present invention may be used to introduce dsRNA into a cell for the treatment or prevention of disease. To treat or prevent a disease or other pathology, a target gene is selected which is required for initiation or maintenance of the disease/pathology. The dsRNA can be introduced into the organism using *in vitro*, *ex vivo* or *in vivo* methods. In an *in vitro* method, the dsRNA is introduced into a cell, which may or may not be a cell of the organism, and the dsRNA-containing cell is then introduced into the organism. In an *ex vivo* method, cells of the organism are explanted, the dsRNA is introduced into the explanted cells, and the dsRNA-containing cells are implanted back into the host. In an *in vivo* method, dsRNA is administered directly to the organism. As noted above, the dsRNA can also be delivered to a cell using one or more vectors that encode the complementary RNAs (or self-complementary RNA), which are then transcribed inside the cell and annealed to yield the desired dsRNA.

In medical applications, the target gene can be an endogenous gene of the organism, or can be the gene of a pathogen. For example, dsRNA may be introduced into a cancerous cell or tumor, and thereby inhibit expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. An exemplary list of potential target genes, including developmental genes, oncogenes, and enzymes, and a list of cancers that can be treated according to the present invention can be found in WO 99/32619 (Fire et al., published 1 July 1999). A candidate target gene derived from a pathogen might, for example,

cause immunosuppression of the host or be involved in replication of the pathogen, transmission of the pathogen, or maintenance of the infection.

The method of the invention can also be used to regulate the expression of an exogenous gene or “transgene” that has been introduced into a host plant or  
5 animal. For example, a transgene that is present in the genome of a cell as a result of genomic integration of the viral delivery construct can be regulated using dsRNA according to the invention.

The present invention allows the creation of plants with reduced susceptibility to climatic injury, insect damage, infection by a pathogen, or with  
10 altered fruit ripening characteristics. In these applications, the targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be  
15 enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The method of the present invention is also useful to identify and  
20 characterize gene function in an organism. In this “functional genomics” approach, dsRNA is targeted to a gene of previously unknown function, and the resultant change in phenotype is observed and, optionally, quantified. This approach is useful to identify potential targets for pharmaceuticals, to promote understanding normal and pathological events associated with development, to  
25 determine signaling pathways responsible for postnatal development and aging, and the like. For example, dsRNA can be designed to target a partial sequence of an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product. As another  
30 example, dsRNA targeted to new genes found by genomic sequencing programs or other “data mining” of genomic data can be used to understand the

physiological roles of these new genes. The ease with which dsRNA can be introduced into an intact cell or organism containing the target gene allows the present invention to be used in high throughput screening (HTS) applications. For example, dsRNA can be produced by an amplification reaction using primers  
5 flanking the inserts of any cDNA or genomic DNA gene library derived from the target cell or organism.

The present invention may be useful in the study of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional  
10 mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

Likewise, if alternative splicing produced a family of transcripts that  
15 were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense  
20 mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit  
25 having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

*Model systems*

Zebrafish. Successful large-scale chemical mutagenesis screens in the zebrafish have led to speculation that this organism will become the vertebrate equivalent of *Drosophila* for the study of developmental genetics (C. Nusslein-Volhard, *Science* 266, 572-574 (1994)). In order for this goal to be realized, as the zebrafish genome is further elucidated, it is imperative to develop techniques for targeted gene knockouts to make maximal use of this vertebrate system. As a vertebrate model, zebrafish has advantages over the mouse including rapid ex-utero development of the relatively transparent embryos, allowing easy access to and visualization of developmental processes. Until now, however, only one technique has been available for targeted interference with gene expression in the zebrafish. This technique employs a ribozyme to mediate a gene "knockdown" (Y. Xie et al., *Proc. Natl. Acad. Sci. U.S.A.*, 94, 13777-13781 (1997)).

Murine NIH/3T3 cells. Murine NIH/3T3 cells are an extremely well-characterized tumor cell line from mice fibroblasts, and have been used to develop and test numerous therapies, including gene therapies, intended for use in humans.

## EXAMPLES

20

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

25

Example I.Double-stranded RNA injection blocks gene expression in zebrafish

To determine whether double-stranded RNA can attenuate endogenous gene expression, single cell zebrafish embryos were injected with double-stranded RNA specifically targeted to three genes of particular interest: GFP, Zf-

30

T and Pax6.1. The phenotypic role played by GFP has been well-characterized in zebrafish; Zf-T is a reporter gene that has been very useful for dissecting promoter activity in zebrafish embryos; and Pax6.1 is a gene that has been thoroughly studied in other organisms.

5

### *Materials*

The GFP expression vector, pEGFP-N1 (GenBank accession number U55762.1), was obtained from Clontech, Inc.

### 10 *Isolation of zebrafish RNA*

RNA from staged zebrafish embryos was obtained using the ULTRASPEC RNA isolation kit (Biotecx Laboratories, Inc.). Fifty embryos are sufficient to obtain the necessary amount of RNA

### 15 *Generation of double-stranded RNA*

Zf-T cDNA fragment. A 321 bp Zf-T cDNA fragment (nucleotide number 1764 to 2085; GenBank accession number S57147) was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA of 8 hour zebrafish embryos. The sequence of upstream primer was 5'

20 TTGGAACAACCTTGAGGGTGA 3' (SEQ ID NO:1) and the downstream primer was 5'CGGTCACCTTTTCAAAGCGTAT 3' (SEQ ID NO:2). To avoid targeting related genes, the primers were designed to amplify a unique portion of the gene that lies outside of the T-box region (Fig. 1).

Ntl insertion sequence cDNA fragment. A 488 bp cDNA fragment of the  
25 *ntl* insertion sequence (GenBank accession number X71596) was also generated by RT-PCR. This fragment disrupts Zf-T in a *ntl* mutant allele and served as a negative control for the double-stranded Zf-T RNA injection. The sequence of the upstream primer was 5' ACCCTATACACCCCCACCTC 3' (SEQ ID NO:3) and the downstream primer was 5' ATAATAGGCACCGCTCATGC 3' (SEQ ID  
30 NO:4).

Pax 6.1 cDNA fragment. A 298 bp Pax6.1 cDNA fragment (GenBank accession number X63183) was generated by RT-PCR of RNA obtained from 24 hour zebrafish embryos. The upstream primer was 5'TTTTCGAGGTTCCCTTGTTG (SEQ ID NO:5) and the downstream primer was 5'AGCCTTTGTATCCTCGCTGA (SEQ ID NO:6). This cDNA fragment lies 5' to the paired box and homeobox.

Nkx cDNA fragments. cDNA fragments for zebrafish Nkx 2-3, 2-5 and 2-7 genes were obtained in a similar manner. For the Nkx 2-3 gene (GenBank accession number U66571), the upstream primer was 5' AACCGTGTTTAACGGGATCA (SEQ ID NO:7) and the downstream primer was 5' GGTGCACTGGCACTACCAT (SEQ ID NO:8), yielding a 291 base pair product, representing positions 775-1065 of the Nkx 2-3 coding sequence. For the Nkx 2-5 gene (GenBank accession number U66572), the upstream primer was 5' CATCTTGCATGCTGTCCACT (SEQ ID NO:9) and the downstream primer was 5'AGATCTTCACCCGGGTCTTC (SEQ ID NO:10), yielding a 250 base pair product, representing positions 232-481 of the Nkx 2-5 coding sequence. For the Nkx 2-7 gene (GenBank accession number U66573), the upstream primer was 5' CATTTGCCAACACGAGTCAA (SEQ ID NO:11) and the downstream primer was 5' CCAGTCCAGTGCCATTTGAT (SEQ ID NO:12), yielding a 141 base pair product, representing positions 911 to 1051 of the Nkx 2-7 coding sequence.

GFP cDNA fragment. A cDNA fragment at the 5' end of the GFP coding sequence was used as template for double-stranded GFP RNA. This 187 bp partial cDNA GFP fragment was obtained using PCR by amplifying a GFP fragment from pEGFP-N1 using chimeric primers containing T7 promoter specific sequence and a GFP sequence. The forward primer was 5'-TAATACGACTCACTATAGGGTAAACGGCCACAAGTTC (SEQ ID NO:13) and the reverse primer was 5'-TAATACGACTCACTATAGGGTCGTGCTGCTTCATGTG (SEQ ID NO:14),

yielding a 187 base pair product representing positions 743 to 930. T7 polymerase was used to simultaneously generate sense and antisense strands using this PCR-generated fragment as a template.

Generation of double-stranded RNA. The Zf-T, Pax6.1, GFP, and Nkx 2-3, 2-5 and 2-7 fragments thus obtained were each individually cloned into pGEM-T vectors containing T7 and SP6 promoters (Promega, Madison, WI). The clones were sequenced to ensure their identity. Double-stranded RNA was then generated from these cloned sequences. To produce the double-stranded RNA, two sets of PCR products were first generated from the cloned fragment (Fig. 1). One PCR product was generated using a primer that is antisense to the T7 promoter sequence, and the other PCR product was generated using a primer that was antisense to the SP6 promoter. The other primers used in these PCRs were specific to the 3' end of the inserted gene fragment relative to the promoter-specific primer being used. PCR products were purified on CENTRICON-100 columns according to manufacturer's instructions (Centricon). T7 RNA polymerase or SP6 RNA polymerase was used to synthesize RNA from each of these templates. *In vitro* transcription reactions were incubated at 37°C for two hours. At completion of each RNA polymerase reaction, RNase-free DNase (e.g., RQ Dnase) was added to the reaction to remove the DNA template. The reaction was incubated at 37°C for an additional 15 minutes, then at 70°C for 10 minutes to inactivate the DNase. RNA was generated in this manner in order to avoid the small amounts of contaminating RNA that can be generated from the opposite strand in RNA synthesis reactions in which plasmids are used as templates. This is especially important for the control experiments since very small amounts of dsRNA can silence gene expression. RNA was purified by centrifugation through a QUICKSPIN column (Boeringer Mannheim); a Sephadex G25 or G50 size exclusion column can also be used. The double-stranded RNA was formed by mixing equal quantities (i.e., 1:1 molar ratios) of the single-strand RNAs that had been denatured at 70°C for 5 minutes in 80 mM



KCl and incubating 1-2 hours at 37°C. Prior to injection, the efficiency of RNA annealing was determined by RNase A (0.5 µg/ml) digestion for 15 minutes at 37°C (Fig. 2).

5 *Microinjection of single cell zebrafish embryos*

Individual single cell embryos were placed in fish embryo water on agarose ramps in petri dishes. Micropipettes are pulled to a fine point and the tip of the pipette is broken using fine tip forceps. The double-stranded RNA is loaded into the micropipette by suction and injected just above the yolk under a  
10 dissecting microscope. Single cell embryos are injected with approximately 10<sup>6</sup> molecules of dsRNA. The injected embryos were subsequently incubated in embryo medium (Westerfield, 1993) at 28.5°C for 12 hours to 5 days.

*RT-PCR*

15 Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to quantify the message level after double-stranded RNA treatment. One hundred zebrafish embryos injected with double-stranded RNA targeted to Zf-T, control double-stranded RNA or uninjected controls were collected at 10 hours. RNA was extracted by RNeasy minicolumn (Qiagen). RNA samples were treated  
20 with 1 unit RNase free-DNase I /1µg RNA, at 37°C for 15 minutes. The RNA was extracted with phenol/chloroform and precipitated with 2.5 volumes of 95% ethanol and 50 ng RNA was used for PCR to confirm that there was no DNA contamination. RNA (0.1µg) was combined with 50 ng of oligo(dT)<sub>15</sub> primers in 10 µl water at 65°C for 10 minutes then cooled to room temperature for 5  
25 minutes. The reverse transcription was carried out at 42°C for 1.5 hours with 0.1µg of total RNA, 0.1 µg of random primer, 20 units RNasin, 200 µM dNTPs, 200 U SuperScript II RNase H reverse transcriptase (GIBCO BRL Inc.), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 20 mM DTT in 50 mM Tris-HCl, pH 8.3, at a final volume of 20 µl. One unit of ribonuclease H was added to digest the RNA at 37°C for 30  
30 minutes. The reaction was stopped by heating at 94°C for 5 minutes.

The PCR was performed in 50 µl containing 2 µl cDNA product from the reverse transcription, 200 µM dNTPs, 10 pmol primers, and 1.0 unit of Taq polymerase, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.001% gelatin in 10 mM Tris-HCl, pH 8.3. A GenAmp PCR System 2400 (Perkin Elmer) was used with the following program: 94°C, 1 minutes, 29 cycles of: 94°C for 25 seconds; 56°C for 20 seconds; 72°C for 30 seconds; final extension was at 72°C for 7 minutes. The PCR product was separated on a 2% agarose gel. The PCR product of the Zf-T gene (GenBank accession number S57147) is 271 bp. It covers the cDNA region of Zf-T from base 1381 to 1750. The forward primer sequence was 5' TTGATCTTGGCTTCAGGAGG 3' (SEQ ID NO:15) and downstream primer was 5' TGCAATGGTTACCAGTTTTGA 3' (SEQ ID NO:16). Primers for zebrafish β actin (used as a control) (GenBank accession number AF 025305) were upstream primer 5' CCCTTGACTTTGAGCAGGAG 3' (SEQ ID NO:17) (starting from base 665) and downstream primer 5' ACAGGTCCTTACGGATGTCG 3' (ending at base 886) (SEQ ID NO:18). The PCR product size was 221 bp.

#### *In situ hybridizations and histology*

*In situ* hybridizations were performed as previously described (Wilkinson, 1992). Sense and antisense probes were generated from a region of the Zf-T gene lying outside the region targeted by the double-stranded RNA. Embryos were fixed in 4% paraformaldehyde at 4°C or Bouin's fixative at room temperature. For histology, embryos were dehydrated in an alcohol series then cleared in xylene. Embryos were embedded in paraffin and sectioned on a microtome at a thickness of 5 µm and were mounted. The slides were deparaffinized, rehydrated and placed in acid alcohol (1% HCl in 70% ethanol) for 5 min, then rinsed in distilled water. Giemsa staining was performed as previously described (Vacca, 1985).

## RESULTS

### *Targeted double-stranded RNA blocks transient expression of GFP*

Fig. 3 shows the effect of double-stranded GFP RNA injection on  
5 transient GFP expression in zebrafish single cell or 16-cell zebrafish embryos. Microinjection of the GFP expression vector pEGFP-N1 into single cell zebrafish embryos resulted in the transient expression of GFP in 85% of the embryos. GFP expression was monitored by fluorescence microscopy throughout early embryogenesis. GFP-targeted double-stranded RNA was  
10 generated for the region shown in Fig. 1, and embryos were co-injected with either GFP double-stranded RNA or a control double-stranded RNA (i.e., the *ntl* insertion sequence dsRNA). When embryos were co-injected with pEGFP-N1 and  $2.9 \times 10^5$  double-stranded RNA molecules, fewer than 3% of embryos had detectable GFP expression. Eighty-four per cent (84%) of embryos co-injected  
15 with control double-stranded RNA showed abundant GFP expression.

When the embryos were injected at the 16-cell stage, injection of pEGFP-N1 alone or pEGFP-N1 with control double-stranded RNA resulted in embryos with scattered, brightly fluorescent cells at 36 hours of development. Co-injection of GFP double-stranded RNA with the pEGFP-N1 completely  
20 quenched the GFP signal.

When Zf-T double-stranded RNA was co-injected with the pEGFP-N1 plasmid into single cell embryos, the embryos had the *ntl* phenotype described below, and showed brilliant GFP expression in scattered cells. These results show that expression of a transiently transfected plasmid can be specifically  
25 attenuated by targeted double-stranded RNA.

### *Targeted double-stranded RNA blocks Zf-T gene expression*

The T gene has dramatic phenotypes in mutant and transgenic animals. The mouse T (Brachyury) gene is required for normal mesoderm development  
30 and extension of the body axis (Herrmann et al., 1990). The zebrafish homologue of the T gene (Zf-T) plays an important role in midline development.

Mutation of the Zf-T gene is known to result in the *no tail (ntl)* mutant phenotype, a Brachyury orthologue. The *ntl* embryos closely resemble mouse T/T mutant embryos in that they lack a differentiated notochord and show poor development of the caudal body (M. Halpern et al., *Cell* 75, 99-111 (1993)).

5        Brachyury encodes a member of the T-box transcription factor family that is expressed in the notochord and is essential for the proper development of midline structures. Loss of function of this gene causes arrested development of notochord. The lack of notochord differentiation leads to disrupted morphogenesis of the mesoderm during gastrulation. This is particularly evident  
10      in the appearance of the somites, which lack the chevron-shaped organization found in wild-type embryos.

Fig. 4 shows the effect of double-stranded Zf-T RNA injection on Zf-T expression in zebrafish single cell embryos. We found 71% of the zebrafish embryos that had been injected at the single cell stage with approximately  $10^4$   
15      double-stranded RNA molecules generated from the Zf-T cDNA fragment had phenotypes that were grossly similar to that of the *ntl* mutant, i.e., truncated tails and disorganized somites (Fig. 4a). Injection of Zf-T single-stranded sense or antisense RNA, or double-stranded RNA generated from the control *ntl* inserted sequence, did not lead to a significant incidence of this phenotype (Table 1).  
20      Simultaneous injection of sense and antisense RNA that were not annealed did not result in a significant incidence of the *ntl* phenotype.

After injection of the Zf-T double-stranded RNA, the Zf-T message was undetectable by *in situ* hybridization in 20% of the embryos (11/56) and weakly expressed in another 50% of embryos injected with Zf-T double-stranded RNA  
25      (Fig. 4b). Semi-quantitative RT-PCR using  $\beta$  actin to control for PCR efficiency and loading, showed an overall 75% reduction in the Zf-T message level from that seen in embryos injected with an unrelated double-stranded RNA.

To determine whether the Zf-T double-stranded RNA had a global effect on gene expression, we co-injected it with pEGFP-N1 into single cell embryos  
30      (Fig. 3, row 4). In these experiments, every embryo that exhibited a *ntl* phenotype also had significant GFP expression. This supports the view that the

phenotypes generated by the injection of double-stranded RNA are not the result of non-specific effects on gene expression.

*Phenocopy of ntl generated by injection of Zf-T double-stranded RNA*

5           To determine whether the mutant zebrafish generated by injection of Zf-T double-stranded RNA into single cell embryos phenocopied the *ntl* mutant, cross-sections of 24 hour embryos injected with Zf-T double-stranded RNA or control double-stranded RNA were examined. The results are shown in Fig. 5. Embryos injected with the Zf-T double-stranded RNA generally lacked a fully  
10   developed notochord as is seen in naturally occurring mutants, while those injected with the control double-stranded RNA had a notochord similar to that seen in uninjected embryos. Twenty-one per cent of the embryos examined that had been injected with Zf-T double-stranded RNA completely lacked notochord (6/28). Somites in the Zf-T attenuated embryos were disrupted in a similar  
15   fashion to that seen in *ntl* zebrafish (Fig. 6). The typical chevron appearance of the somites was lacking, but somites were not fused across the midline as is seen in *floating head* mutants.

20           *Effect of Zf-T double-stranded RNA on the expression of sonic hedgehog and floating head*

          The expression patterns of *shh* and *flh*, two genes that are also essential to proper midline development in zebrafish, were examined by *in situ* hybridization. Fig. 7 shows the effect of Zf-T double-stranded RNA injection on the expression of *shh*. In 27 % of the embryos examined (15/26), the expression  
25   pattern of *shh* throughout the floorplate of the embryos injected with the Zf-T double-stranded RNA was 3-4 cells wide. This is identical to the expression pattern found for this gene in *ntl* embryos (Halpern et al., 1997). More than 50% of the embryos examined had a similar, but less complete alteration of *shh* expression. In the embryos injected with the control double-stranded RNA, *in*  
30   *situ* hybridization showed *shh* expression limited to a one-cell stripe along the midline as is found in wild-type embryos.

In wild-type zebrafish embryos, *flh* is expressed in the anterior and posterior nervous system and in a narrow axial strip. The effect of Zf-T double-stranded RNA injection on the expression of *flh* is shown in Fig.8. In 33% of the embryos examined (6/18), expression of *flh* in the Zf-T double-stranded RNA injected embryos was unaffected in the anterior and posterior nervous system but was greatly diminished or absent along the axis. Embryos injected with Zf-T double-stranded RNA also show diffuse and broadened *flh* expression in the tailbud. This is similar to the expression pattern of *flh* found in the *ntl* mutant (A. Melby et al., *Dev. Dyn.* 209, 156-165 (1997)). This partial effect on *flh* expression was observed in more than 80% of the embryos examined.

*Dose-response for generation of the ntl phenotype*

To determine the number of double-stranded RNA molecules required to generate a phenotype, single cell embryos were injected with approximately 1 nl of a solution containing double-stranded Zf-T RNA concentrations ranging from  $1.6 \times 10^5$  to  $5.0 \times 10^8$  molecules of double-stranded RNA/nl. Phenotype was determined for each embryo at 48 hours post-injection. As can be seen in Table 1, embryos that had been injected with  $10^6$  or more Zf-T double-stranded RNAs exhibited a very high incidence of the *ntl* phenotype. A grossly complete phenotype was observed in more than 20% and a partial phenotype was observed in 50% of these embryos (Table 1; Fig. 9). Embryos injected with  $4.0 \times 10^6$  double-stranded RNAs or less did not show a significant incidence of the *ntl* phenotype. Embryos injected with a control double-stranded RNA were phenotypically normal. Embryos injected simultaneously with sense and antisense single-stranded RNAs also did not display abnormal phenotypes.

Table 1. The injected RNA molecular numbers and embryos with phenotypic changes.

	Injection	Molecules(1) (X 10 <sup>6</sup> )	Number of embryos	Viable embryos	Phenotypic change		
					None	Partial	Full
5	Uninjected	0	296	239	239	0	0
	S ssRNA(2)	100	768	621	619	2(0.6%)	0
	A ssRNA(3)	100	715	583	580	3(0.5%)	0
10	S/A ssRNA(3)	100	708	587	585	2(0.3%)	0
	C dsRNA(4)	100	959	815	810	5(0.6%)	0
	Zf-T dsRNA						
		0.16	550	468	466	2(0.4%)	0
		0.8	ND	79	73	4(5%)	2(3%)
15		4	328	288	275	11(4%)	2(0.7%)
		20	257	229	196	23(10%)	10(4%)
		50	161	129	79	38(29%)	12(9%)
		100	1975	1618	455	839(51%)	322(20%)
		290	769	531	93	275(51%)	163(31%)
20		500	1206	822	97	438(53%)	287(35%)

(1) The injected RNA numbers for each embryo.

(2) Single-stranded RNA of sense (S) and antisense (A) direction.

(3) S/A indicates the unannealed sense and antisense RNA mixture.

25 (4) Control (C) double-stranded RNA.

Thus, microinjection of double-stranded Zf-T RNA resulted in a high incidence of a phenotype similar to that of *ntl*. Furthermore, Zf-T gene expression could not be detected by *in situ* hybridization and the message was  
30 decreased by 75% as monitored using semiquantitative RT-PCR in 12 hour

embryos that had been injected with the double-stranded RNA. Expression of the zebrafish genes *sonic hedgehog* and *floating head* were altered in the embryos microinjected with the Zf-T double-stranded RNA in a manner that is remarkably similar to the zebrafish *no-tail* mutant.

5

*Targeted double-stranded RNA blocks Zf-Pax6.1 gene expression*

Another unique and dramatic phenotype is associated with a naturally occurring Pax6 mutation, which was found in the mouse mutant small eyes. These embryos lack the lens placodes and normal forebrain structures. Zebrafish  
10 Pax6.1 transcripts can first be detected in the presumptive forebrain and hindbrain regions of the neural plate. Expression has also been observed in the optic vesicles and lens placodes, confirming that the Pax6.1 protein is expressed in those areas of the eye where it is assumed to control differentiation. These expression patterns correlate well with a role for Pax6.1 in lens placode and  
15 brain development in zebrafish. A second closely related gene, Zf-Pax6.2, has an expression pattern that overlaps with that of Pax6.1 in zebrafish embryos.

As shown in Fig.10, injection of double-stranded RNA of the zebrafish Pax6.1 cDNA fragment resulted in embryos with grossly underdeveloped heads and absent or greatly diminished eyes. Expression of Pax6.1 message was absent  
20 in embryos injected with double-stranded RNA, but undiminished in embryos injected with *ntl* double-stranded RNA. Twenty-five percent of the embryos injected with the Zf-Pax6.1 double-stranded RNA exhibited phenotypes. Thus, microinjection of double-stranded RNA targeted to Pax6.1 was associated with depressed expression of Pax6.1 and resulted in absent or greatly reduced eye and  
25 forebrain development, similar to the phenotype seen in mouse mutants.

*Targeted double-stranded RNA blocks Zf-Nkx 2-7 gene expression*

Nkx2-5 was identified by virtue of its homology to the *Drosophila* gene tinman. Null expression of tinman is associated with absent development of the  
30 *Drosophila* dorsal vessel, which is similar in some ways to the vertebrate heart. A number of members of the Nkx gene family play crucial roles in normal



vertebrate heart development. Several Nkx family members are expressed in the developing heart of one or more vertebrate species, including Nkx 2-3, 2-5, 2-6, 2-7, and 2-8. Hemizygous mutations in the human Nkx2-5 gene, for example, are located on chromosome 5q34, are associated with defective cardiac septation and congenital heart block. Three Nkx family members known to be expressed in the zebrafish heart field: Nkx2-3, Nkx2-5, and Nkx2-7.

In this experiment, embryos that were injected with Zf-Nkx 2-7 double-stranded RNA exhibit altered heart morphology and the hearts function poorly.

#### 10 *Targeted silencing of multiple genes*

As shown in Fig. 11, embryos that were injected simultaneously with Zf-T and Zf-Pax6.1 double-stranded RNA exhibited defective development of both the tail and head, combining the phenotypic defects associated with mutations of either gene alone. Examination of cross-sections through the head confirmed that eye and brain structures were defective in embryos injected with Zf-T double-stranded RNA. Cross-sections through the tail region showed that notochord was lacking or greatly diminished and somites were disorganized. There was a greater incidence of defects in the tail region (greater than 90%) than in the eye or head (25%). This demonstrates that multiple genes can be simultaneously targeted for diminished expression by injection of targeted double-stranded RNAs.

#### DISCUSSION

We have now shown that targeted gene silencing can be accomplished in a vertebrate embryo by injection of double-stranded RNA into single cell embryos. This method allowed us to disrupt the activity of specific genes encoding the zebrafish homologue of Brachyury (Zf-T), zebrafish Pax6.1 and the reporter gene, GFP. We also have shown that multiple genes can be targeted simultaneously using this method. In addition, embryos that were co-injected

with a GFP expression vector and Zf-T double-stranded RNA developed *ntl* phenotypes while GFP expression was unaffected, showing that the effects of the Zf-T double-stranded RNA are not non-specific.

Depending on the timing and/or amount of dsRNA injected, partial phenotypes of varying severity can be generated. In cases where null phenotypes are particularly severe, this allows the identification of effects that would ordinarily be missed. That is, by permitting gene silencing at later stages of development, the technique has allowed us to explore the effects of blocking the expression of a gene whose inhibition would be lethal at an earlier developmental stage. This has allowed us to unmask potential roles for Zf-T in developmental processes in which it had not been previously implicated. The most severe phenotypes mirror those found in animal mutant models, such as the zebrafish *ntl* mutant, naturally occurring mutation in Zf-T, and the mouse mutant small eyes, which is deficient in Pax6 expression.

Functionally attenuating expression of Zf-T resulted in a reproducible phenotype that mirrored that found of the *ntl* mutant where the same gene was altered by an insertional mutation. Interestingly, we were able to use this inserted sequence as a negative control for these experiments as it produced no phenotype. By a number of criteria, more than 20% of all embryos injected with the Zf-T double-stranded RNA developed in a manner that phenocopied the *ntl* mutant. Zf-T gene silencing produced by injection of double-stranded RNA was apparent at the message level and by the specific phenotypes that were generated. In the zebrafish embryos, co-injection of unannealed sense and antisense RNA strands did not result in a high percentage of mutants. This is in contrast to results obtained in the nematode where phenotypes could be obtained when the sense and antisense strands were injected separately (A. Fire et al., *Nature* 391, 806-810 (1998)).

None of the control treatments, i.e., single strand sense or antisense, or control ds RNA showed any phenotypes at any concentrations that were tested. The only phenotypes we observed were after injection of Zf-T or Pax6.1 double-stranded RNA, and these phenotypes specifically matched the treatment. In this

regard double-stranded RNA targeted to Pax6.1 affected head and eye development with no apparent effect on tail development (Fig. 12). On the other hand Zf-T ds RNA affected tail development without any apparent effect on the head or eye (Fig. 4). Thus this treatment does not behave like a non-specific, toxic effect which would be expected to yield similar phenotypes regardless of the double-stranded RNA injected.

GFP, a reporter gene encoding a protein that fluoresces when exposed to ultraviolet light, allows promoter activity to be monitored in living embryos throughout development. Using this reporter gene, it has been possible to identify promoter regions that regulate gene expression in the zebrafish notochord, sympathetic neurons, and hematopoietic lineages. We microinjected single-cell embryos with a plasmid vector containing GFP regulated by a promoter that drives ubiquitous expression during early development. We found that co-injection of double-stranded RNA specifically targeted to GFP suppressed expression of this reporter gene in well over 95% of the embryos injected with the plasmid vector.

While it might at first be thought that the GFP plasmid vector would be a good marker for the distribution of double-stranded RNA, the differences in these two nucleotides might predict great differences in their processing by the embryo for two reasons. The GFP plasmid is approximately 5000 base pairs of deoxyribonucleotides while the double-stranded RNA is composed of ribonucleotides and it is a very short piece by comparison (around 200 base pairs). Data from *C. elegans* (Montgomery et al., 1998) and our own observations indicate that the double-stranded RNA has much freer access to the cell from the extracellular matrix than does the GFP expressing plasmid. Whether this is because of the difference in nucleotide composition or size is not known.

The injection of Zf-T double-stranded RNA into single cell zebrafish embryos resulted in greatly diminished expression of the Zf-T as monitored by *in situ* hybridization and semi-quantitative RT-PCR, and resulted in phenotypes very similar to those found in *ntl* zebrafish embryos. Our *in situs* of *ntl* are

shown at 9 hours. Most of the published *ntl in situs* show embryos at 11 hours or later and the signal is stronger than what we have shown in Fig. 4. However, it seems clear from this figure that the *ntl* expression is completely absent after the double-stranded RNA injection and correlates perfectly with the phenotypes of the older embryos. This visual result is substantiated by semiquantitative RT-PCR showing that the Zf-T message is reduced to 70% of control level. It should be emphasized that semiquantitative RT-PCR was done using pooled zebrafish embryos and the message levels would represent the whole range of phenotypes shown in Fig. 9. Thus a 70% reduction in message level seems quite reasonable.

Not only is the phenotype grossly similar to *ntl*, it is quite specific. In more than 70% of the embryos injected with the double-stranded Zf-T RNA, the notochord was absent or greatly diminished. Twenty-one per cent of the embryos examined had no notochord. The somites in these embryos also lacked the characteristic chevron appearance observed in wild type embryos, similar to the *ntl* mutant. The *ntl* phenotype can be distinguished from that of another tailless phenotype seen in the *floating head* mutant by the lack of fusion of the somites across the midline. Embryos injected with Zf-T double-stranded RNA did not show fusion of the somites across the midline providing a differential diagnosis for the *ntl* versus the similar floating head phenotype.

That these phenotypic characteristics were due to specific attenuation of Zf-T expression was supported by *in situ* hybridizations showing altered expression patterns of *shh* and *flh*. Zf-T expression was greatly diminished in 30-50% and absent in 20% of the embryos injected with the Zf-T double-stranded RNA. Expression of *shh* and *flh* were also altered in the zebrafish midline in these embryos in a manner similar to that found in the *ntl* mutant. The expression patterns of these genes appeared to be identical to those found in the *ntl* mutant in approximately 25% of the microinjected embryos that were examined. The expression patterns were altered in a similar, but less complete manner, in more than 60 % of the microinjected embryos.

Twenty-five percent of zebrafish embryos that were injected with Zf-Pax6.1 double-stranded RNA had underdeveloped heads and absent or greatly diminished eyes. *In situ* hybridizations confirmed that Pax6.1 expression was greatly diminished in more than 90% of the embryos injected with Zf-Pax6.1 double-stranded RNA. Pax6.1 expression was not affected in embryos injected with a control double-stranded RNA. The relatively low occurrence of phenotypes in embryos injected with Pax6.1 double-stranded RNA may be due to functional redundancy of the closely related Pax6.2, which is expressed in overlapping regions of the zebrafish embryo. Interestingly, simultaneous injection of double-stranded RNA targeted to Pax6.1 and *ntl* gives a very clear compound phenotype that is quite distinct from the phenotypes resulting from injection of either double-stranded RNA separately. We believe that this data along with that from simultaneous injection of GFP expression vector with Zf-T double-stranded RNA in which the effect appeared completely independent forms a compelling argument to support the idea that treatment with double-stranded RNA causes a relatively specific cellular response.

Finally, it is known that certain types of double-stranded RNA, such as mismatched or polyI/polyC RNA, can be toxic at high concentrations in eukaryotic animals (M. Kumar et al., *Microbiol. Mol. Biol. Rev.* 62, 1415-1434 (1998)). Although double-stranded RNA can induce interferon- $\alpha/\beta$  in non-immune cells, this toxicity is primarily due to an immune system response mediated through interferon production in response to viral infections. Immune system or interferon- $\alpha/\beta$  -mediated toxicity is very unlikely to play any role in generating the phenotypes we have observed. First, the phenotypes that we have generated can be observed in 24 hour embryos, long before the zebrafish immune system has been established. The thymus primordium appears in the zebrafish at approximately 54 hours, but does not enlarge significantly until 30 hours later. Rag1 and Rag2 expression cannot be detected until day 4, indicating a lack of mature T cells in the zebrafish until that time. Second, the amount of double-stranded RNA that was used to generate the phenotypes is much less than is necessary to cause this interferon-mediated cell toxicity (M. Kumar et al.,

*Microbiol. Mol. Biol. Rev.* 62, 1415-1434 (1998)). We have also found that polyI/polyC RNA can be toxic both in cultured 3T3 cells and in microinjected embryos. However, none of the ten double-stranded RNAs that we have so far examined elicit a toxic effect *in vitro* or *in vivo*. Third, the phenotypes that have been generated for each gene under study differ substantially from one another and are specifically related to the gene that was targeted. Finally, injection of control double-stranded RNA at the same concentrations does not cause a detectable deviation from the wild type expression levels or phenotype.

In summary, these results show that double-stranded RNA can efficiently disrupt gene activity in zebrafish. This inhibitory activity appears to be specific to the targeted gene. Non-specific double-stranded RNA had no apparent phenotypic effect. We have also shown that multiple genes can be simultaneously targeted.

## Example II.

### Double-stranded RNA injection blocks gene expression in explanted cardiac neural crest tissue

Attenuated expression of HirA (GenBank accession number X99375) is known to be associated with increased persistent truncus arteriosus (PTA). Double-stranded RNA was generated from a chick HirA cDNA fragment in essentially the same manner as described for zebrafish in Example I. The upstream primer was 5' TCTGCACCAGCATTAGCACT (SEQ ID NO:19) and the downstream primer was 5' TGCTGTGAGAATTCGACTGG (SEQ ID NO:20) yielding a 201 base pair product representing positions 2095 to 2295 of the HirA cDNA sequence.

Explanted chick neural crest was incubated for 1.5 hours with HirA dsRNA or nonspecific dsRNA (control *ntl* dsRNA) in DMEM. The concentration of double-stranded RNA applied was approximately  $10^6$  molecules per nanoliter. Following exposure to the double-stranded RNA each piece of tissue was washed in PBS prior to placing the tissue into culture.

Fig. 12 shows that double-stranded HirA RNA effectively silences expression of HirA in cardiac neural crest cell culture. Using RT-PCR as described in Example I, it was determined that the HirA message was decreased by 58%. This inhibitory activity appears to be specific to the targeted gene.

5 Non-specific double-stranded RNA had no apparent effect.

### Example III.

#### Double-stranded RNA injection blocks gene expression in mammalian cell culture

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Double-stranded GFP RNA was prepared as described in Example I. Murine NIH/3T3 cells were transfected with pEGFP-N1 and double stranded GFP RNA using a standard transfection procedure. First, murine NIH/3T3 cells (~2x 10<sup>8</sup> per well) were seeded in a six-well tissue culture plate in 2 ml of DMEM with 10% FBS. The cells were then incubated at 37°C in a CO<sub>2</sub> incubator until they were about 70-80 % confluent (i.e., 18-24 hours).

15

For each transfection, Solution A was made by diluting 1 µg of pEGFP-N1 and 0.5-1 µg of double stranded GFP RNA into 100 µl serum-free medium (OPTI-MEM® 1 Reduced Serum Medium, GIBCO BRL Cat. No. 320-1985), and Solution B was made by diluting 3-12 µl of LIPOFECTAMINE Reagent into 100 µl serum-free medium. For one control experiment, Solution A contained the plasmid pEGFP-N1 but no double stranded RNA; for another, Solution A contained the plasmid pEGFP-N1 a control double-stranded *ntl* RNA. The two solutions were combined, mixed gently, and incubated at room temperature for 30 minutes. The cells were washed once with 2 ml of serum-free DMEM. For each transfection, 0.8 ml of serum-free DMEM was added to each tube containing the lipid-DNA complexes. The tubes were mixed gently and the diluted complex solution was overlayed onto the washed cells. The cells were incubated for 5 hours at 37°C in a CO<sub>2</sub> incubator. DMEM (1 mL) was added with 20 % FBS without removing the transfection mixture. Medium was replaced at 18-24 hours following the start of transfection. Cell extracts were

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assayed for GFP activity 24-72 hours after the start of transfection. GFP expression was monitored by fluorescence microscopy.

As shown in Fig. 13, transformed cells incubated with double-stranded GFP RNA molecules exhibited substantially reduced GFP expression. In  
5 contrast, transformed cells incubated with control dsRNA showed no apparent change in GFP expression.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (e.g., GenBank amino acid and  
10 nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for  
15 variations obvious to one skilled in the art will be included within the invention defined by the claims.



What is claimed is:

1. A method for attenuating the expression of a target gene in a cell comprising introducing into the cell a double stranded RNA in an amount sufficient to attenuate expression of the target gene, wherein the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene.
2. The method of claim 1 wherein the target gene is an endogenous gene.
3. The method of claim 1 wherein the target gene is a foreign gene.
4. The method of claim 1 wherein the targeted gene is a chromosomal gene.
5. The method of claim 1 wherein the targeted gene is an extrachromosomal gene.
6. The method of claim 1 wherein the targeted gene is derived from a pathogen capable of infecting the cell.
7. The method of claim 6 wherein the pathogen is selected from the group consisting of a virus, bacterium, fungus or protozoan.
8. The method of claim 1 wherein the cell is a vertebrate cell.
9. The method of claim 8 wherein the vertebrate cell is a fish cell.
10. The method of claim 8 wherein the vertebrate cell is a mammalian cell.
11. The method of claim 10 wherein the mammalian cell is a murine cell.

12. The method of claim 10 wherein the vertebrate cell is an avian cell.
13. The method of claim 1 wherein the cell is an invertebrate cell.
14. The method of claim 1 wherein the cell is a plant cell.
15. The method of claim 1 wherein the double stranded RNA comprises a nucleotide sequence that is completely identical to the nucleotide sequence of at least a portion of the target gene.
16. The method of claim 1 in which the essentially identical nucleotide sequence is at least 50 bases in length.
17. The method of claim 1 wherein the double stranded RNA is administered in an amount sufficient to completely inhibit expression of the target gene.
18. The method of claim 1 in which the double stranded RNA comprises one strand which is self-complementary.
19. The method of claim 1 in which the double stranded RNA comprises two separate complementary strands.
20. The method of claim 1 wherein the cell is an embryo.
21. The method of claim 20 wherein the embryo is a fish embryo.
22. The method of claim 20 wherein the double stranded RNA is introduced into the embryo using microinjection.

23. The method of claim 1 wherein the cell is present in a cell culture, a tissue, an organ, or an organism.
24. The method of claim 23 wherein the cell is present in an organism, and the double stranded RNA is introduced into a body cavity or interstitial space of the organism.
25. The method of claim 23 wherein the cell is present in an organism, and wherein the double stranded RNA delivered to the organism via oral, topical, parenteral, vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration.
26. The method of claim 23 wherein the cell is present in a cell culture or a tissue explant, and wherein introduction of the double stranded RNA into the cell comprises incubating the cell culture or tissue explant in a solution comprising the double stranded RNA.
27. The method of claim 1 wherein the double stranded RNA is treated with RNase prior to its introduction into the cell.
28. The method of claim 1 further comprising, prior to introducing the double stranded RNA into the cell, annealing two complementary single stranded RNAs to yield the double stranded RNA.
29. The method of claim 1 wherein the complementary single stranded RNAs are annealed in the presence of potassium chloride.
30. The method of claim 1 wherein the function of the target gene is unknown.
31. The method of claim 1 further comprising introducing into the cell a second double stranded RNA in an amount sufficient to attenuate expression of a second

target gene, wherein the second double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the second target gene.

32. The method of claim 1 comprising introducing into the cell multiple double stranded RNAs in an amount sufficient to attenuate expression of multiple target genes, wherein each double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of a target gene.

33. A method for attenuating the expression of a target gene in an organism comprising:

introducing a double stranded RNA into an embryo in an amount sufficient to attenuate expression of the target gene, wherein the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene;  
growing the embryo into an adult organism in which expression of the target gene is attenuated.

34. The method of claim 33 wherein the organism is a vertebrate.

35. The method of claim 33 further comprising identifying a phenotypic change in the organism associated with attenuated expression of the target gene.

36. The method of claim 33 wherein expression of the target gene in the organism is completely inhibited.

37. A method for attenuating the expression of a target gene in cell culture comprising:

introducing a double stranded RNA into a cell in an amount sufficient to attenuate expression of the target gene, wherein the double stranded RNA

comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene; and  
culturing the cell to yield a cell culture in which expression of the target gene is attenuated.

38. The method of claim 37 wherein the cell is a vertebrate cell.

39. The method of claim 37 further comprising identifying a phenotypic change in the cell culture associated with attenuated expression of the target gene.

40. The method of claim 37 wherein expression of the target gene in the cell culture is completely inhibited.

41. A method for attenuating the expression of a target gene in a tissue explant comprising:

explanting a tissue from an organism; and

introducing a double stranded RNA into a cell of the tissue explant in an amount sufficient to attenuate expression of the target gene, wherein the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene.

42. The method of claim 41 wherein the tissue explant exhibiting attenuated expression of the target gene is implanted back into the organism.

43. The method of claim 41 wherein the tissue explant exhibiting attenuated expression of the target gene is implanted into a second organism.

44. The method of claim 41 wherein the tissue is fetal tissue.

45. The method of claim 41 wherein the organism is a vertebrate.

46. The method of claim 41 further comprising identifying a phenotypic change in the tissue explant associated with attenuated expression of the target gene.

47. The method of claim 41 wherein expression of the target gene in the organism is completely inhibited.

48. A method for attenuating the expression of a target gene in a cell comprising:

annealing two complementary single stranded RNAs in the presence of potassium chloride to yield double stranded RNA;

contacting the double stranded RNA with RNase to purify the double stranded RNA by removing single stranded RNA; and

introducing the purified double stranded RNA into the cell in an amount sufficient to attenuate expression of the target gene;

wherein the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene.

49. A method for treating or preventing a disease or infection in a mammal comprising:

identifying a target gene, wherein expression of the target gene is associated with the disease or infection; and

administering to the mammal a double stranded RNA in an amount sufficient to attenuate expression of the target gene;

wherein the double stranded RNA comprises a nucleotide sequence that is essentially identical the nucleotide sequence of at least a portion of the target gene.

50. The method of claim 49 for treating or preventing a viral infection, wherein the double stranded RNA is an antiviral double stranded RNA that attenuates the expression of a viral gene.

51. The method of claim 49 for treating or preventing cancer, wherein the double stranded RNA is an antitumor double stranded RNA.

52. The method of claim 49 for treating an autosomal dominant genetic disease wherein the double stranded RNA attenuates the expression of an allele of a gene that is associated with the disease.

53. The method of claim 52 wherein the disease is Huntington's chorea.

54. The method of claim 49 comprising:  
identifying a plurality of target genes, wherein expression of the target genes is associated with the disease or infection; and  
concurrently administering to the mammal a plurality of double stranded RNAs in an amounts sufficient to attenuate expression of the target genes;  
wherein each double stranded RNA comprises a nucleotide sequence that is essentially identical the nucleotide sequence of at least a portion of a target gene.

55. The method of claim 49 further comprising:  
identifying a second target gene, wherein expression of the second target gene is associated with a second disease or infection; and  
administering to the mammal a second double stranded RNA in an amount sufficient to attenuate expression of the second target gene concurrent with the administration of the first double stranded RNA;  
wherein the second double stranded RNA comprises a nucleotide sequence that is essentially identical the nucleotide sequence of at least a portion of the second target gene.

56. A method for reducing or preventing the rejection response to transplant tissue comprising administering to the transplant tissue a double stranded RNA that attenuates the expression of a gene in the transplant tissue that can elicit an immune response in the recipient.

57. The method of claim 56 wherein the transplant tissue is hepatocytes.

58. A vertebrate cell comprising a double stranded RNA comprising a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of a target gene.

59. The vertebrate cell of claim 58 selected from the group consisting of a fish cell, a murine cell, a bird cell and a human cell.

60. A vertebrate comprising the cell of claim 58.

61. A kit comprising reagents for attenuating the expression of a target gene in a cell, the kit comprising:

a DNA template comprising two different promoters selected from the group consisting of a T7 promoter, a T3 promoter and an SP6 promoter, each promoter operably linked to a nucleotide sequence, such that two complementary single stranded RNAs are capable of being transcribed from the DNA template, and wherein the complementary single stranded RNAs comprise a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene;

a plurality of primers for amplification of the nucleotide sequence;

nucleotide triphosphates for forming RNA;

at least two RNA polymerases, each capable of binding to a promoter on the DNA template and causing transcription of the nucleotide sequence to which the promoter is operably linked;

a purification column for purifying single stranded RNA;

buffer for annealing single stranded RNAs to yield double stranded RNA; and

RNase A or RNase T for purifying double stranded RNA.



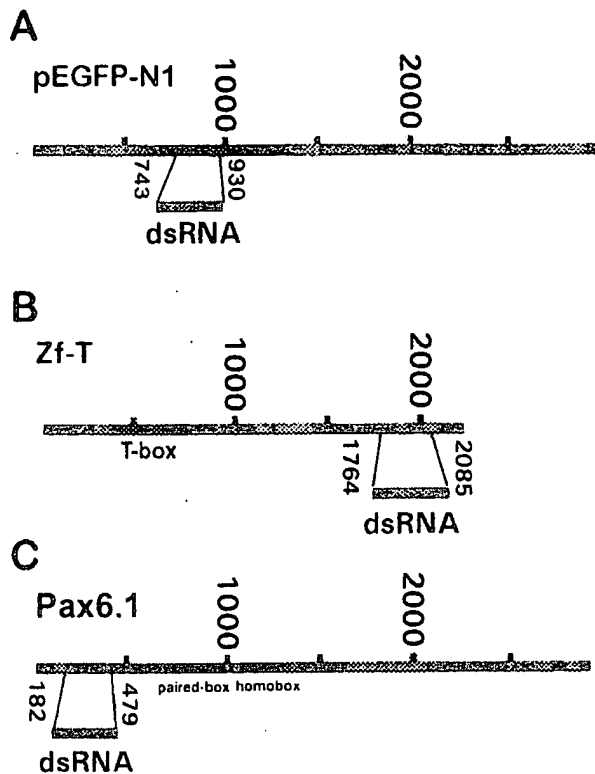


Fig. 1

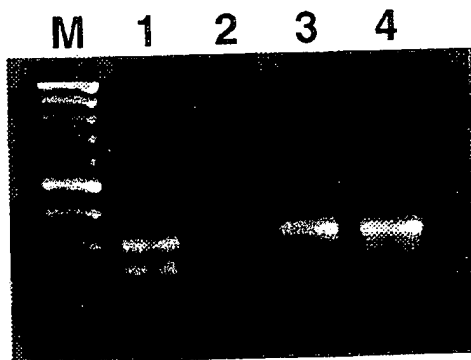


Fig. 2

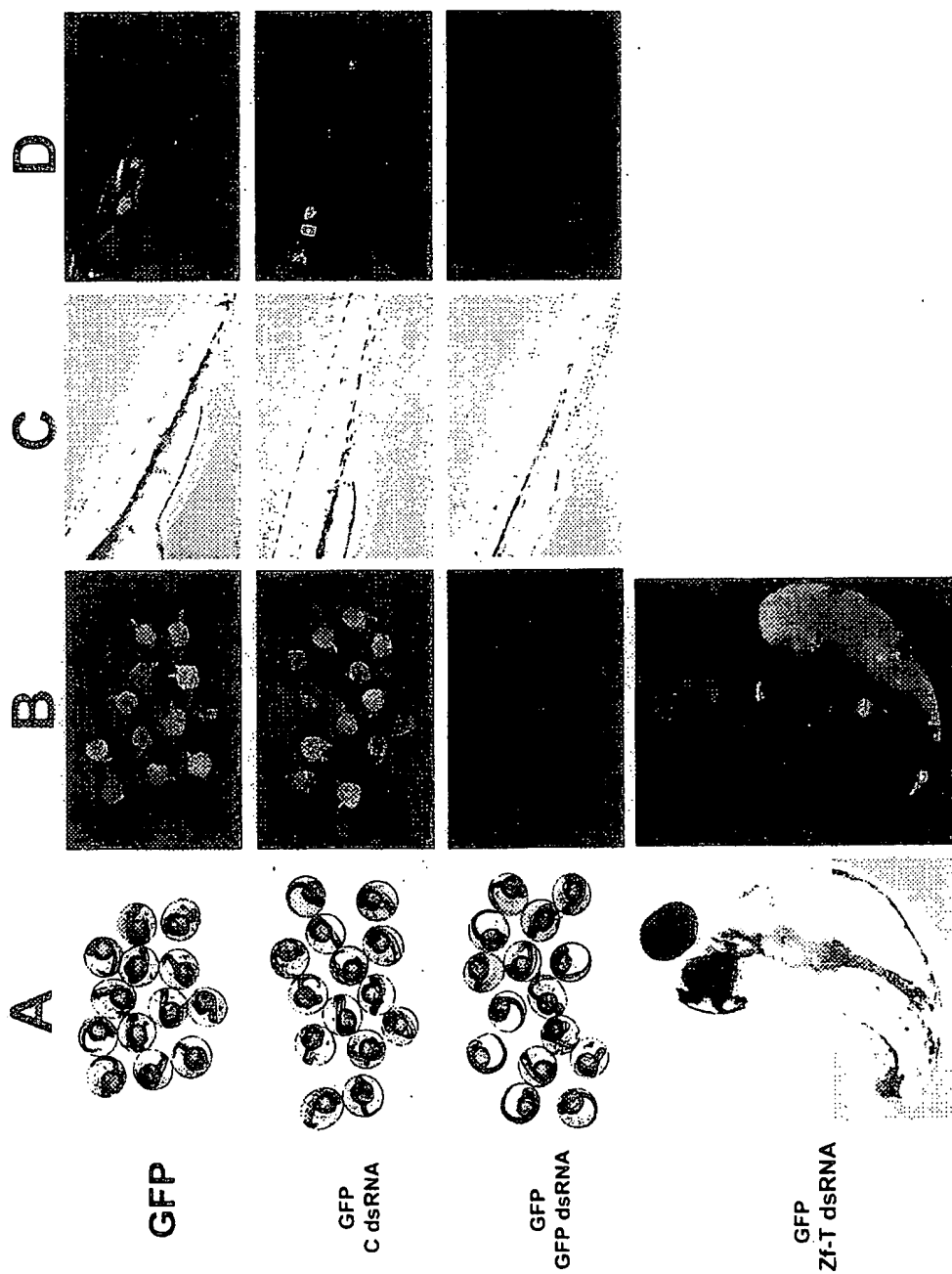


Fig. 3

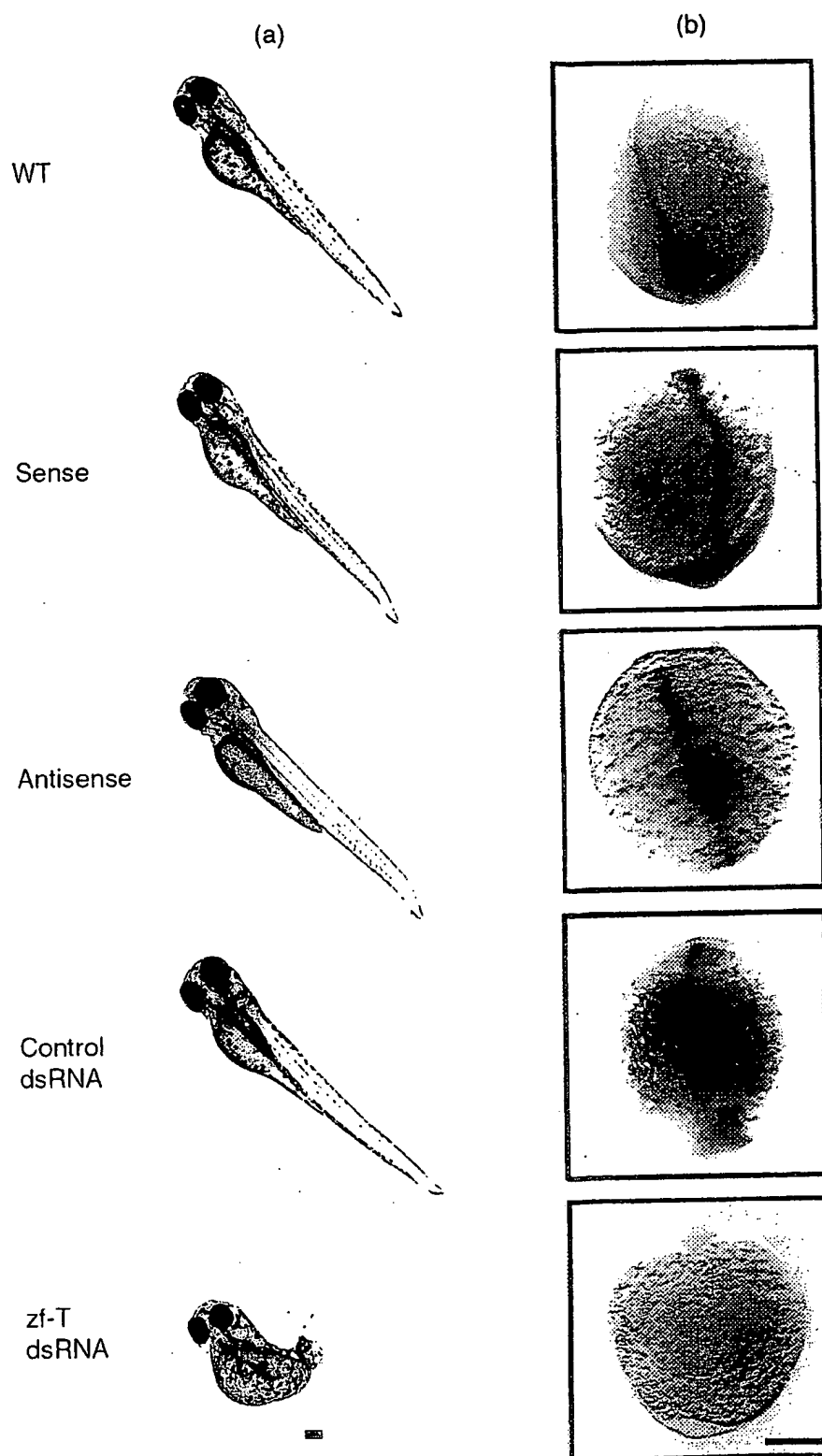


Fig. 4

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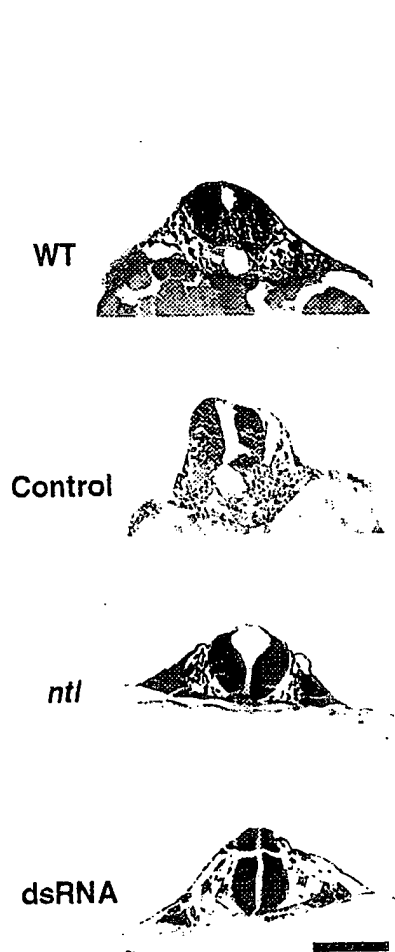


Fig. 5

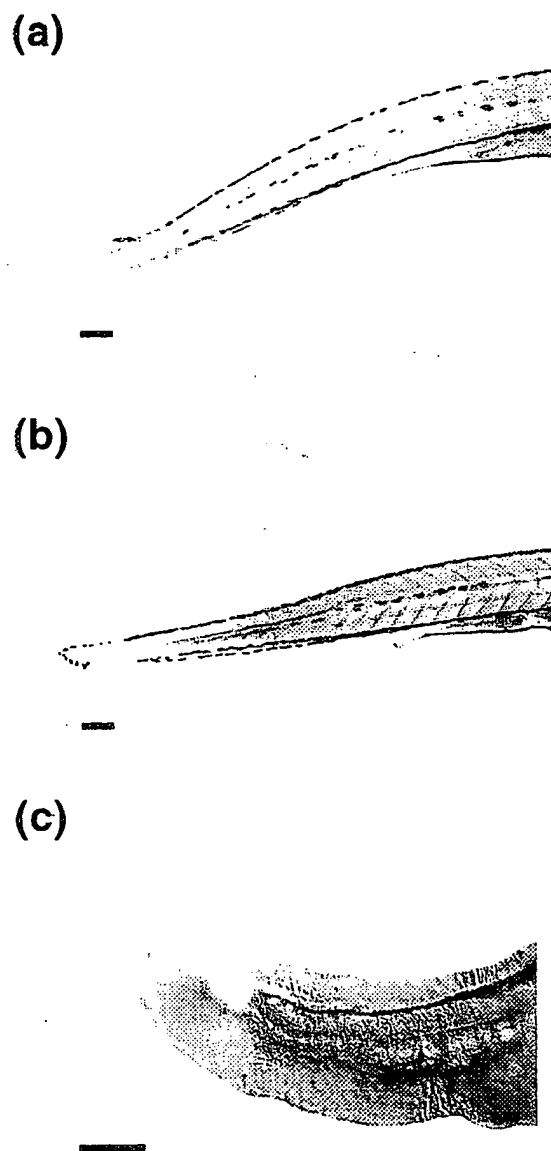


Fig. 6

Fig. 7

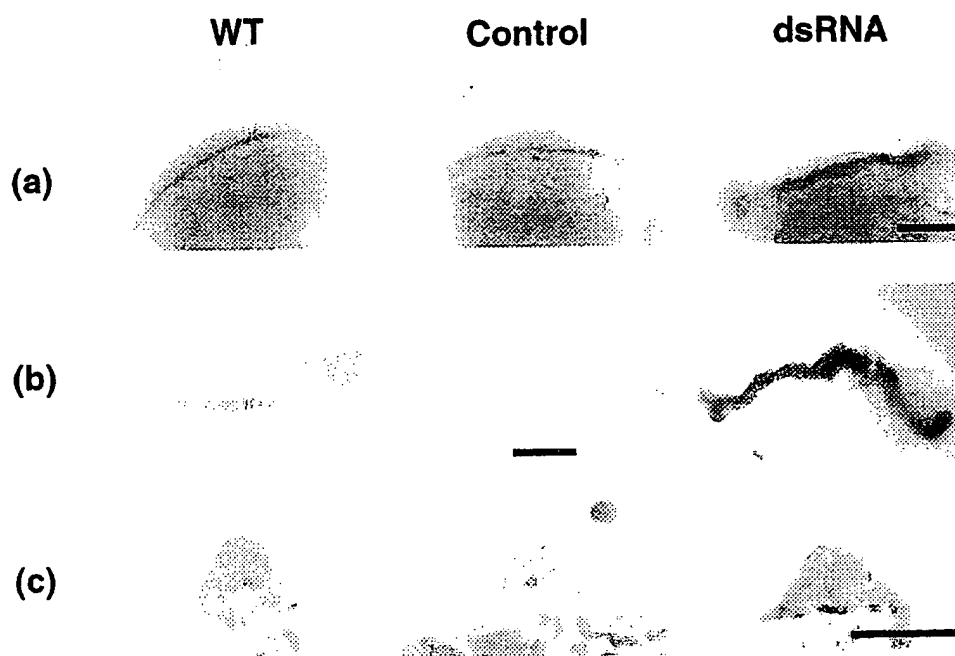
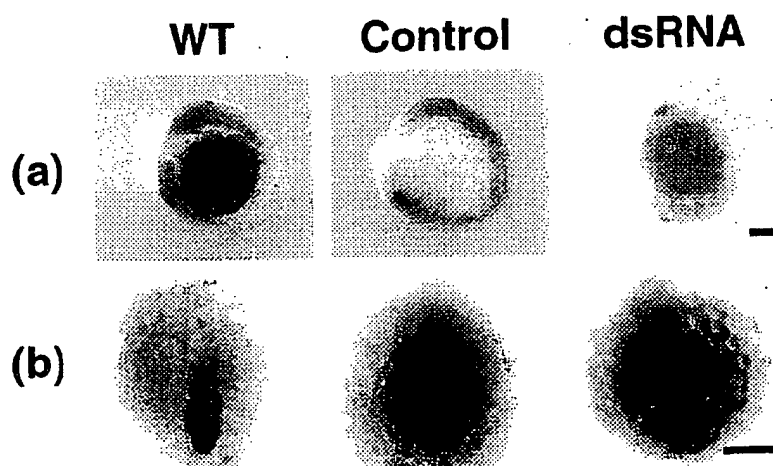


Fig. 8



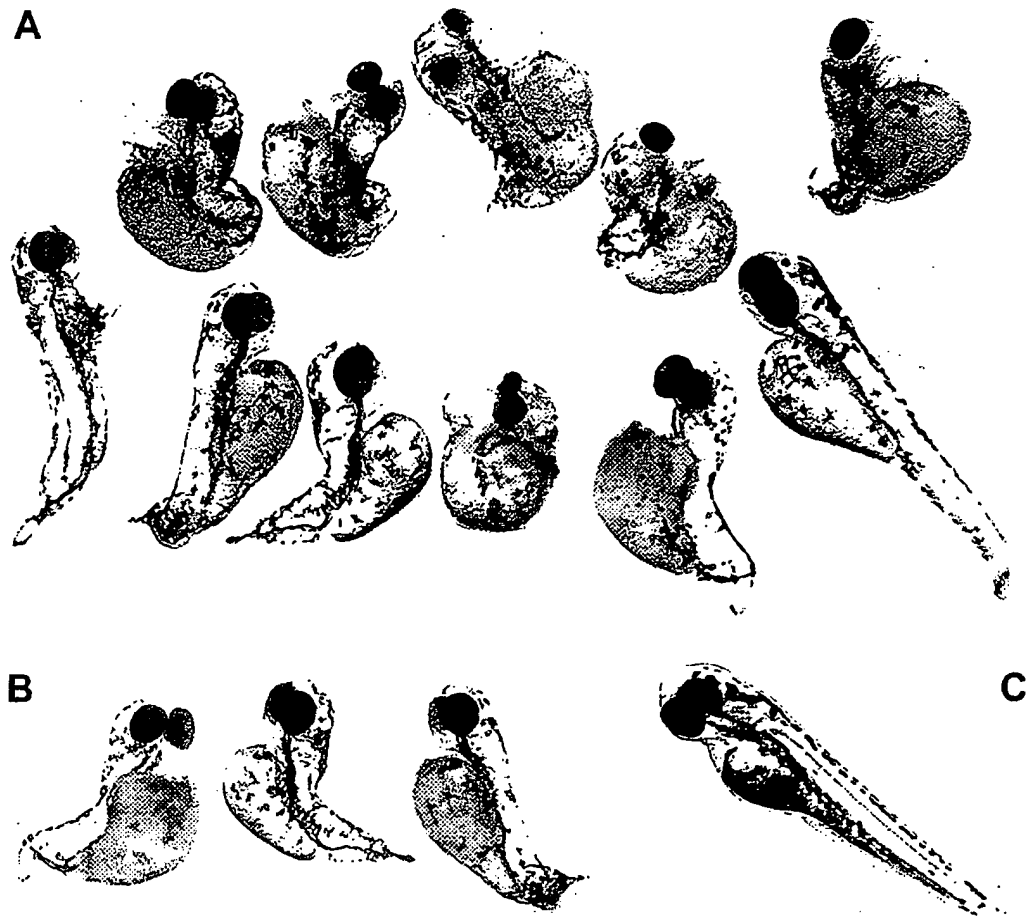


Fig. 9

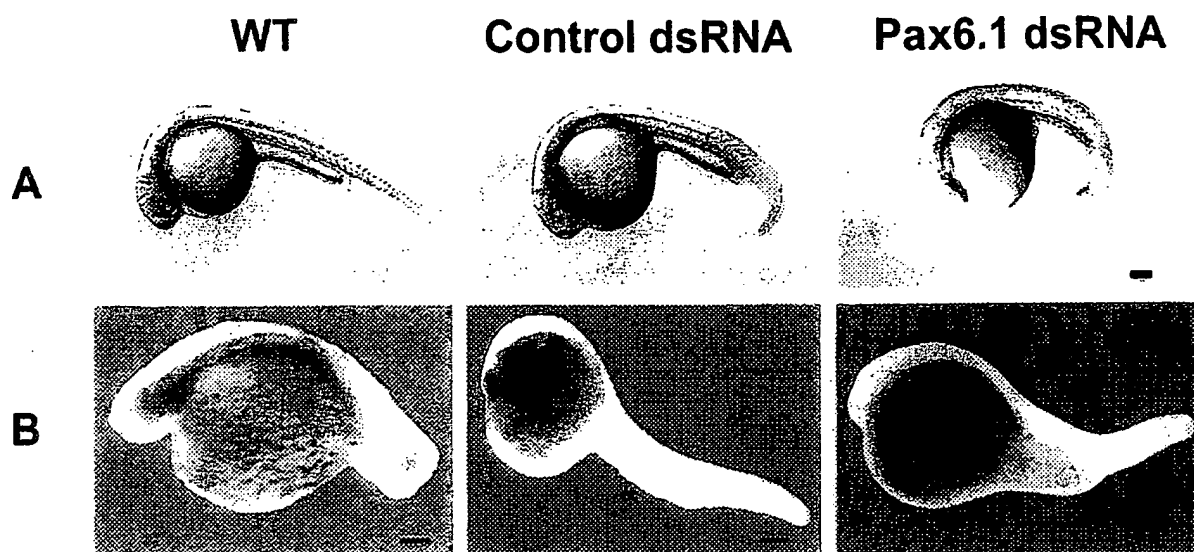


Fig. 10

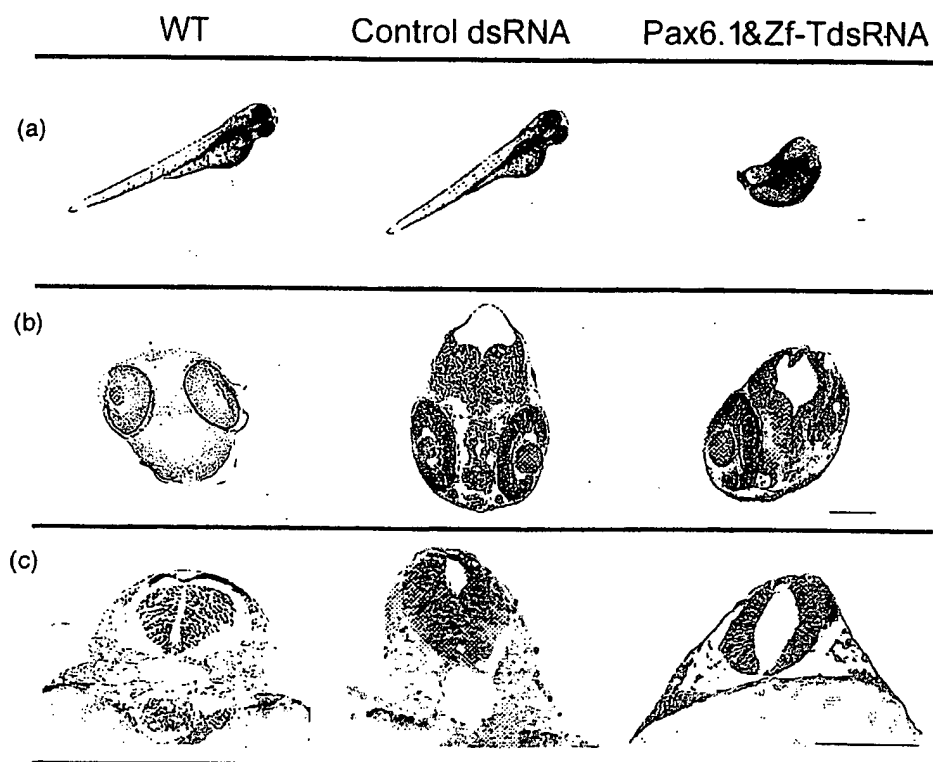


Fig. 11

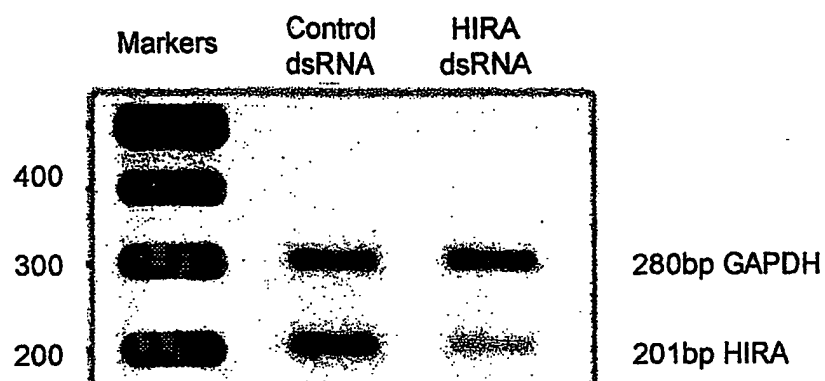


Fig. 12



## dsRNA effect on Mice NIH/3T3 Cell Line

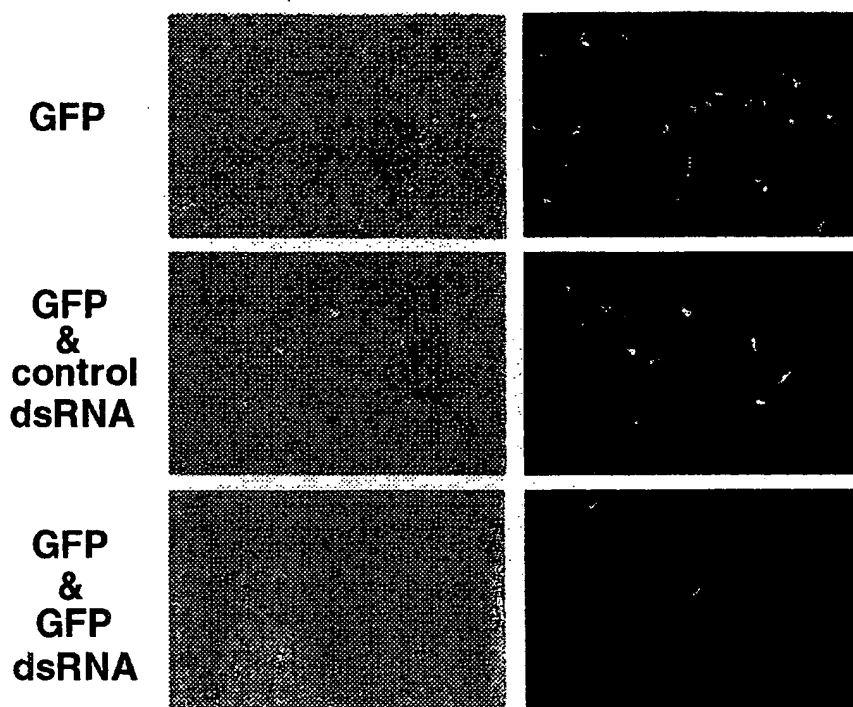


Fig. 13

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/02227

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N15/63 A01K67/027 A61K31/713

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLIN BIOSIS EPO-Internal EXTERNAL DATABASES

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONTGOMERY, M. ET AL.: "RNA as a target of double-stranded RNA-mediated genetic interference in <i>Caenorhabditis elegans</i> ". PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 95, December 1998 (1998-12), pages 15502-15507, XP002138441 ISSN: 0027-8424 cited in the application	1-5,13, 15,17, 19, 23-25, 28,29
Y	the whole document	27,48
Y	US 5 422 241 A (GOLDRICK MARIANNA ET AL) 6 June 1995 (1995-06-06) column 9, line 65 -column 10, line 21 claims	27,48
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

23 May 2000

Date of mailing of the international search report

09/06/2000

Name and mailing address of the ISA

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Authorized officer

Andres, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/02227

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WATERHOUSE ET AL: "Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, November 1998 (1998-11), pages 13959-13964, XP002114472 ISSN: 0027-8424 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1,3,5-7, 14,15, 17-19, 23,50</p>
X	<p>NGÔ, H. ET AL.: "Double-stranded RNA induces mRNA degradation in Trypanosoma brucei"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 95, December 1998 (1998-12), pages 14687-14692, XP002138442 ISSN: 0027-8424 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1,2,4,6, 7,13,15, 19,23, 28,49</p>
X	<p>FIRE A ET AL: "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans"</p> <p>NATURE, vol. 391, 19 February 1998 (1998-02-19), pages 806-811, XP002095876 ISSN: 0028-0836 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1-5,13, 15,17, 19,23, 24,28, 35,36</p>
X	<p>TIMMONS L ET AL: "Specific interference by ingested dsRNA"</p> <p>NATURE, vol. 395, no. 6705, 29 October 1998 (1998-10-29), page 854 XP002103601 ISSN: 0028-0836 the whole document</p> <p style="text-align: center;">---</p>	<p>1,2,4, 13,15, 17,19, 23-25</p>
P,X	<p>WO 99 32619 A (CARNEGIE INST OF WASHINGTON ;MONTGOMERY MARY K (US); FIRE ANDREW ( ) 1 July 1999 (1999-07-01) cited in the application</p> <p style="text-align: center;">---</p> <p>the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-11, 13-19, 23-26, 28,30, 35,36, 49-51, 58-60</p>

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/02227

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WARGELIUS A. ET AL.: "Double-stranded RNA induces specific developmental defects in zebrafish embryos." BIOCHEM BIOPHYS RES COMMUN 1999 SEP 16;263(1):156-61, XP002138443 the whole document	1-5,8,9, 15,17, 19-24, 33-36, 58-61
P,X	LI YX. ET AL.: "Double-stranded RNA injection produces null phenotypes in zebrafish." DEV BIOL 2000 JAN 15;217(2):394-405, XP002138444 the whole document	1-5,8,9, 15, 19-24, 31-36, 58-61
P,X	WIANNY F. ET AL.: "Specific interference with gene function by double-stranded RNA in early mouse development." NAT CELL BIOL 2000 FEB;2(2):70-5, XP002138445  *** published 23-12-1999 (see p. 74) *** the whole document	1-4,8, 10,11, 15,17, 19,20, 22,23, 33-36, 58-61
P,X	WO 99 61631 A (NOVARTIS ERFINDUNGEN VERWALTUN ;NOVARTIS AG (CH); HEIFETZ PETER BE) 2 December 1999 (1999-12-02)  page 1, last paragraph -page 13 examples 1-5 claims	1-5,14, 15, 17-19, 23,28, 30,33, 35,36

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 00/ 02227

### Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. .tional Application No

PCT/US 00/02227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5422241 A	06-06-1995	NONE	
WO 9932619 A	01-07-1999	AU 1938099 A	12-07-1999
WO 9961631 A	02-12-1999	AU 4368399 A	13-12-1999



October 10, 2005

Micron Technology, Inc.  
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William J. Baer, Esq.  
Arnold & Porter LLP  
555 Twelfth Street, NW  
Washington, DC 20004-1206

Dear Mr. Baer,

As of September 23, 2005, you, as our counsel, furnished by letter to our independent accountants, PricewaterhouseCoopers LLP, Attention: Gordon S. Lee, 10 Almaden, Suite 1600, San Jose, CA 95113, information concerning litigation, claims and assessments discussed in our letter of September 14, 2005 to which you have devoted substantive attention on behalf of Micron Technology, Inc. in the form of legal consultation or representation. This request is limited to contingencies amounting to \$500,000 individually or items involving lesser amounts that exceed \$500,000 in the aggregate for this purpose.

Please confirm to PricewaterhouseCoopers LLP that the statements made in your letter as of September 23, 2005 continue to be correct as of the effective date of your reply to this letter or furnish to them such explanation, if any, that you consider necessary to supplement the information previously furnished to them, including information regarding any pending or threatened litigation, claims and assessments not commented on previously by you.

We also have advised PricewaterhouseCoopers LLP that since September 23, 2005 there are no unasserted claims or assessments if there are additional unasserted claims or assessments insert, "except as set forth below", that you have advised are probable of assertion and must be disclosed or considered for disclosure in accordance with Statement of Financial Accounting Standards No. 5.

To facilitate the evaluation of your response by our independent accountants, please respond by November 1, 2005. Your response should include matters that existed as of September 1, 2005 during the period from that date to the effective date of your response. They would appreciate receiving your reply by that date with a specified effective date no earlier than October 31, 2005.

Very truly yours,

A handwritten signature in black ink, appearing to read "Roderic W. Lewis".

Roderic W. Lewis  
V.P., Legal Affairs, General Counsel  
& Corporate Secretary

*The future of memory*